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*J Immunol* 1999; 162:1723-1729;
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A Transgenic Model to Analyze the Immunoregulatory Role of IL-10 Secreted by Antigen-Presenting Cells

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IL-10 is a cytokine secreted by a wide variety of cell types that has pleiotropic stimulatory and suppressive activities on both lymphoid and myeloid cells in vitro. To analyze the consequences of high IL-10 secretion by APCs in immune responses, we produced transgenic mice expressing human IL-10 directed by the MHC class II Ea promoter. Despite alterations in the development of T and B cells, no gross abnormalities were detected in peripheral lymphocyte populations or serum Ig levels. However, when immunized using conditions that give either a Th2-type or a Th1-type response, IL-10 transgenic mice failed to mount a significant T or B cell immune response to OVA. IL-10 transgenic mice were also highly susceptible to infection with intracellular pathogens like Listeria monocytogenes or Leishmania major, in contrast to IL-10 transgenic mice, where the transgene was expressed in T cells. Finally, the recently described stimulatory effect of IL-10 on CD8+ T cells was confirmed by the ability of IL-10 transgenic mice to limit the growth of immunogenic tumors by a CTL-mediated mechanism. These results demonstrate, that depending on the type of immune response, IL-10 can mediate immunosuppressive or immunostimulatory activities in vivo. The Journal of Immunology, 1999, 162: 1723–1729.

IL-10 is a cytokine secreted by a wide variety of cell types that has pleiotropic stimulatory and immunosuppressive properties in mouse and human in vitro assays (1). For example, IL-10 has been shown to prevent Ag-specific T cell proliferation and cytokine production indirectly by reducing the Ag-presenting capacities of monocytes (2, 3). This effect is associated with down-regulation of class II MHC molecules (4) and costimulatory molecules such as B7.1, B7.2, and ICAM-1 (5, 6). IL-10 also potently suppresses many effector functions of monocytes and macrophages, including the release of proinflammatory monokines and chemokines (7, 8) and the production of NO4 (9).

In addition to inhibiting Ag-specific responses, we have shown that IL-10 induces long lasting Ag-specific anergy in human CD4+ T cells (10) and that repetitive Ag-specific activation of both human and mouse CD4+ T cells in the presence of IL-10 leads to the differentiation of a new subset of regulatory cells called T regulatory cells 1 (Tr1) (11). These suppressive and antiinflammatory activities of IL-10 suggest its potential clinical use as an immunosuppressant in allogeneic transplantation and autoimmune diseases (12). However, some studies have shown IL-10 unable to suppress an immune response in vivo. For example, IL-10 was not able to prevent (13), and in some situations even exacerbated, graft-vs-host disease (14) when administered either at the same time or after the bone marrow transplant. The discrepancy between these in vivo effects of IL-10 may be due to the fact that IL-10 also stimulates activated CD8+ T cells (15).

To determine the effects of increased IL-10 production specifically by APCs in vivo, we generated IL-10 TG mice (TG+) in which the human IL-10 cDNA was regulated by the mouse MHC class II Ea promoter and expressed only by MHC class II-positive cells. Human IL-10 is fully active in the mouse, but the species difference allows the transgene encoded, but not host IL-10, to be specifically neutralized by anti-hIL-10 Abs. With these TG mice, the consequences of elevated IL-10 in the differentiation of T cells and the induction of tolerance were evaluated by well-characterized immunization protocols. The impact of chronic exposure to IL-10 and its effect on monocyte-mediated protection were analyzed in two intracytoplasmic models of infection, Listeria monocytogenes and Leishmania major. Finally, the potential immunostimulatory role of IL-10 in tumor rejection was analyzed in the P815 melanocytoma model.

Materials and Methods

Mice

The class II MHC Ea promoter sequences (16) (Ea position −2172 to +12) was cloned upstream of a human IL-10 cDNA sequence. The construct was injected into eggs from BALB/cJ females, and TG founders were backcrossed three generations to BALB/cAnN mice. Presence of the transgene was confirmed using Southern blot analysis with a probe encompassing the human IL-10 cDNA and by PCR with human IL-10-specific cDNA primers. TG mice on the BALB/cAnN, (DBA2 × BALB/c)F1, (C57BL/6 × BALB/c) background were bred at DNAx (Palo Alto, CA). Control mice were either nontransgenic littermates or were purchased from Simonsen Laboratories (Gilroy, CA).

Cell lines, culture, and reagents

All assays were conducted in Yssel’s medium (17) supplemented with 10% FCS. Mice were immunized with 10 μg/mice of OVA (Sigma, St. Louis, MO) with either alum (Sigma) injected i.p. or with CFA (Sigma) injected at the base of the tail. Spleen cells suspension were treated with 0.83% ammonium chloride to lyse red blood cells, washed, and resuspended in

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Received for publication April 24, 1998. Accepted for publication October 26, 1998.

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4 Abbreviations used in this paper: NO, nitric oxide; Tr1, T regulatory cells 1; TG, transgene, hIL-10, human IL-10; HPRT, hypoxanthine phosphoribosyltransferase; mIL-10, murine IL-10; LmAg, Leishmania major Ag.
culture medium. CD4+ T cells were purified from the spleen or lymph nodes of mice by negative depletion using anti-B220, anti-Mac-1, and anti-CD8 mAbs (PharMingen, San Diego, CA) and sheep anti-rat-coated Dynabeads (Dynabeads, Robbins Scientific, Mountain View, CA). Resident peritoneal macrophages were collected as described (8), and contaminant B and T cells were removed with anti-B220, anti-CD4, and anti-CD8 MAb (PharMingen) and sheep anti-rat-coated Dynabeads.

The P815 (H-2b) mastocytoma cell line, derived from a DBA/2 mouse, is highly tumorigenic in syngeneic mice. OVA was purchased from Sigma and used at 500 μg/ml in proliferative and cytokine assays. The neutralizing anti-hIL-10 9D7 was previously described (18).

**PCR analysis**
cDNA synthesis was performed as described previously (19). PCR analysis was conducted as described previously (19). PCR cycles were 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, with 35 cycles using the primers TGG CAA CCT TAA CCA for HPRT. PCR cycles were 30 s at 94°C, and 30 s at 60°C, with 30 cycles using the primers

**Cytokine ELISAs**
Cytokine levels in supernatants were detected by two-site sandwich ELISAs as previously described for mIFN-γ (20), mIL-4 and mIL-10 (18), and hIL-10 (10). Samples were assayed in duplicate and quantitated by comparison with standard curves of purified recombinant or natural cytokine.

**Ig isotype ELISAs**
Serum samples were assayed for Ig concentration by a two-site sandwich ELISA according to the manufacturer’s instruction (Pierce, Rockford IL) and OVA-specific Ig isotype as described (21).

**Flow cytometry**
For analysis, splenocytes were stained with FITC or phycoerythrin (PE)-conjugated mAbs (PharMingen). Flow cytometry analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

**Parasite infections and L. major antigen preparation**
L. major (WHO strain designation WHOM/173), a kind gift from Dr. R. Locksley, were cultured as promastigotes in M199 (Life Technologies, Grand Island, NY) containing 5% FCS (J. R. Scientific, Woodland, CA), 2 mM t-glutamine, and 100 U/ml each of penicillin and streptomycin. Promastigotes were harvested from stationary phase cultures and washed in PBS. Mice were infected with 1.5 × 106 viable promastigotes, injected s.c. into the left hind footpad. L. major Ag, used for in vitro restimulations (LmAg) was prepared by four cycles of freezing and thawing of the parasites, followed by centrifugation. The Ag preparation was added to culture wells at an equivalent of 2 × 107 organisms/ml. Parasite loads were determined by limiting dilution culture of cell suspensions from lesion-draining lymph nodes. The remainder of each lymph node suspension was used for in vitro stimulation of CD4+ T cells as described below.

**Recall responses to LmAg in vitro**
CD4+ T cells were purified from lesion-draining popliteal lymph nodes and restimulated with LmAg (equivalent to 2 × 107 organisms/ml) and nontransgenic BALB/c splenic APC. APC were prepared by depletion of CD4+ and CD8+ T cells from spleen cell suspensions followed by 1000 rad of γ-irradiation. CD4+ T cells (5 × 106/ml) and APC (2 × 106/ml) were cultured with Ag for 72 h, and the supernatants were harvested for assay of cytokines.

**L. monocytogenes infection**
Mice were injected i.v. with 5 × 106 live L. monocytogenes organisms. For the death curves, each group contained five animals, which were monitored daily for survival. Anti-hIL-10 mAb (9D7) was administered at day −1, day 0, and day 7 (1 mg/mouse i.p.).

**Tumor production**
P815 cells (1 × 106) were injected s.c. in a volume of 0.1 ml into the right flanks of DBA/2 × BALB/cF1 IL-10 TG or nontransgenic mice. Tumor size was measured every 3 days as the product of tridimensional parameters.
IL-10 has been shown to down-regulate the expression of co-stimulatory molecules at the surface of monocytes/macrophages. However, no difference in the expression of MHC molecules, ICAM-1, or CD86 molecules was observed in splenic macrophages from IL-10 TG compared with nontransgenic littermates (Table I).

**Table I.** No major modification in splenocytes of hIL-10 transgenic mice

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<th>Mouse Type</th>
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<th>CD4⁺ (%)</th>
<th>CD8⁺ (%)</th>
<th>Ratio CD4/CD8</th>
<th>B220⁺, IgM⁺</th>
<th>B220⁺, CD23⁺</th>
<th>B220⁺, CD69⁺</th>
<th>Mac1⁺, ICAM-1⁺</th>
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<td>(10 ± 1)</td>
<td>(15 ± 1)</td>
<td>(10 ± 1)</td>
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*Cells from spleen of hIL-10 transgenic (TG) or control littermate mice (CT) were isolated, counted and stained with the appropriate Abs. Cell number are given as million of cells, and values are given as percentages of total gated lymphocyte populations for T and B cell markers or as total MAC-1⁺ cells for macrophages.

**FIGURE 2.** Mitogen-induced T cell proliferation and Ig levels are not altered in hIL-10 TG mice. *a,* Total splenocytes from hIL-10 TG or control littermate mice were isolated and cultured for 3 days with medium alone or stimulated with immobilized anti-CD3 mAb (1 μg/ml in PBS for 4 h; gray bars) or Con A (1 μg/ml; black bars). The proliferative responses were measured by [³H]TdR incorporation during the last 12 h of culture. One of four experiments is shown. *b,* Serum samples from hIL-10 TG mice (filled circles) and control littermate (open circles) mice were assayed for the indicated Ig isotypes by ELISA. Each symbol represents data from one individual mouse.

**hIL-10 TG mice have a defective response to OVA**

To determine the effect of altered IL-10 expression in the TG mice on T cell differentiation in vivo, mice were immunized with OVA in the presence of two different adjuvants: alum, which favors a Th2 response, or CFA, which favors development of a predominant Th1 response. One week after the second weekly injection of OVA in the presence of alum or CFA, the amounts of the different Ig isotypes specific for OVA were measured (Fig. 3a). Reduced OVA-specific Ab responses were observed in the IL-10 TG mice, as compared with nontransgenic littermates, regardless of the type of adjuvant used (Fig. 3a). This inhibition of both Th1- and Th2-type responses in IL-10 TG mice was confirmed by the analysis of the proliferative response or the production of cytokines by either splenocytes or draining lymph node cells from immunized mice. For example, no significant proliferative response was observed in purified CD4⁺ T cells from IL-10 TG mice immunized with OVA in alum after restimulation in vitro with OVA presented by irradiated wild-type BALB/c splenocytes (Fig. 3b). In contrast, purified CD4⁺ T cells from nontransgenic littermates immunized with OVA in alum proliferated after in vitro restimulation (Fig. 3b). Similarly, the IFN-γ levels observed after in vitro restimulation of CD4⁺ T cells purified from popliteal lymph nodes of mice immunized with OVA in CFA were greatly reduced in the IL-10 TG mice as compared with the nontransgenic siblings (Fig. 3c).

**IL-10 TG mice have enhanced susceptibility to L. monocytogenes**

To investigate the role of IL-10 in bacterial infections, IL-10 TG mice and control animals were infected with varying doses of *L. monocytogenes*. The IL-10 TG animals displayed a 100% mortality rate within 2 wk of inoculation with 5 × 10⁴ bacteria, a dose that was sublethal in wild-type animals (Fig. 4). The sensitivity of the IL-10 TG mice was completely reverted by the administration of anti-hIL-10 mAb (at day −1, day 0, and day 7 of infection; Fig. 4) whereas IL-10 TG mice treated with an isotype control Ab remained susceptible (not shown).

**IL-10 TG mice fail to control L. major infection**

The consequence of overproduction of IL-10 on chronic infection with an intracellular pathogen was examined in mice infected with the protozoan parasite, *L. major*. Cutaneous infection of resistant
Mouse strains induces a highly polarized Th1 response that leads to resolution of the localized lesion and resistance to infection (23). The principal effector mechanism in mice is the IFN-\(\gamma\)-dependent production of NO by macrophages. Since IL-10 is both a potent inhibitor of IFN-\(\gamma\) production by T cells as well as NO production by macrophages, higher levels of IL-10 might be expected to inhibit control of parasite growth and enhance disease progression. Since BALB/c mice are themselves highly susceptible to \(L.\) major infection, TG BALB/c mice were crossed with resistant C57BL/6J to produce a resistant F1 background. TG CB6F1 mice developed progressive footpad lesions that closely resembled the lesions in susceptible BALB/c mice (not shown), whereas nontransgenic littermates were quite resistant to the infection (Fig. 5). The greatly enhanced susceptibility to \(L.\) major was completely reversed by...
treated the mice with a neutralizing anti-hIL-10 Ab beginning 1 day before the infection, demonstrating that the TG IL-10 was acting during infection rather than acting on lymphocyte development before the infection.

Direct evidence for impaired parasite killing in TG mice was obtained by estimating parasite numbers in the draining lymph node 4 wk after infection. TG mice had 200-fold more parasites than nontransgenic controls (Table II). Although high parasite counts and progressive lesion development are characteristic of mice with a predominant Th2 response to *L. major*, T cells from both TG and nontransgenic CB6F1 mice produced similar, Th1-like cytokine responses to LmAg in vitro (Table II).

**Biphasic effect of IL-10 expression on the antitumor reactivity**

The role of IL-10 in tumor rejection and the generation of CTL responses remains controversial, and both positive or negative effects have been reported. To analyze the CTL response against tumor cells in IL-10 TG mice we used the immunogenic P815 H-2d mastocytoma. In syngeneic DBA/2 or in (DBA/2 × BALB/c)F1 mice, P815 is a highly tumorigenic mastocytoma cell line. Tumor-associated transplantation Ags present in P815 have been identified and are able to induce CTL responses in syngeneic mice that specifically kill P815 in vitro (24). In spite of this, the antitumor response was not fully effective, and the tumors grew progressively, resulting in death within 20–30 days (Fig. 6). Treating mice with anti-CD8 mAb resulted in a more acute and rapid growth of the tumor in vivo (Fig. 6a). In IL-10 TG mice, injection of P815 resulted in a very rapid growth of the tumor cells in the first 2 wk. Injection of the anti-hIL-10 mAb 24 h prior to tumor cell injection enabled the IL-10 TG mice to control the growth of the transplanted tumors, whereas delayed injection of anti-hIL-10 Abs (at day 10) had no effects in controlling the growth of the tumor (Fig. 6b).

However, in untreated IL-10 TG mice after 3 wk, the tumor cells were completely eliminated whereas the tumor continued to grow in the nontransgenic controls and in IL-10 TG mice treated after 10 days with anti-hIL-10 Abs. This late protective mechanism observed in IL-10 TG mice seems to be mediated, in part, by IL-10-activated CD8+ T cells since treatment of IL-10 TG mice with anti-CD8 mAb or anti-hIL-10 injected after 10 days resulted in the development of a tumor growth pattern similar to that observed in anti-CD8 mAb-treated nontransgenic siblings.

**Discussion**

Here, we describe an in vivo model to assess the consequences of elevated IL-10 on the regulation of different immune responses. The MHC class II Ea promoter directs the expression of human IL-10 only in APCs displaying the MHC class II molecules. The expression of IL-10 by stromal cells that express MHC class II molecules impaired the maturation of T cells in the thymus (M.
early as 7 days after infection. At the dose used in these experiments, 5 × 10^7 organisms/ml, one-half of the TG mice died within 2 wk and all were dead 4 wk after infection. This time course suggests inhibitory effects on both the innate and Ag-specific components of the response to *Listeria* and is consistent with the previously reported inhibition of the innate response by recombinant IL-10 (25). Treatment with anti-hIL-10 beginning just before infection reversed this increased susceptibility, demonstrating that it was a direct result of hIL-10 produced during the course of the infection.

In a more chronic model of infection, using the protozoan parasite, *L. major*, TG IL-10 also caused a marked inhibition of parasite control in genetically resistant CB6F1 mice. This was suggested by the progressive development of the footpad lesion and was confirmed by direct measurements of the parasite burden in the lesion-draining lymph node. Although the lesion progression and parasite counts were similar to those usually seen in highly susceptible BALB/c mice (not shown), the actual T cell response was quite different. Instead of the predominantly Th2 cytokine response of BALB/c mice, CB6F1 Tg^−/− mice developed a Th1-like response similar in magnitude to the nontransgenic controls. That this Th1 response was not able to control the infection in TG mice strongly suggested that IL-10 was acting primarily to inhibit the microbicidal effector functions of this Th1 response. This view was confirmed by the demonstration that anti-hIL-10 Ab treatment could reverse this increased susceptibility. Preliminary experiments suggest that lesion development in TG mice can be changed from progressive to resolving, even in mice not treated with anti-hIL-10 mAb until 4 wk after infection (data not shown). Thus, high levels of IL-10 in vivo are capable of inhibiting Th1 responses to such an extent as to render ineffective a normally protective Th1 response to these intracellular pathogens. A recent description of a different IL-10 TG mouse showed no significant inhibition of control of an infection with *L. major* (27). A possible difference is that the IL-10 transgene in that report was controlled by the IL-2 promoter, and there could be significant differences in the levels of IL-10 produced in these two mice, especially in the case of a chronic infection.

The injection of tumorigenic P815 cells into IL-10 TG mice showed a biphasic action of IL-10. In the initial phase, the growth of the tumor was not controlled in IL-10 TG mice as compared with nontransgenic siblings. This mechanism was inhibited by the addition of anti-hIL-10 Abs at day −1 and reflected the inhibitory activities of IL-10 as previously observed in the IL-2 promoter-driven mIL-10 TG mice (27). However, in a second phase, when the tumor expanded rapidly in the nontransgenic littermates, it was completely rejected in hIL-10 TG mice. This second phase seemed to rely on the activation of CTL since no rejection of the tumor was observed in IL-10 TG mice previously treated with depleting anti-CD8 mAb or treated with anti-hIL-10 Abs after 10 days. Indeed, the results concerning the effects of IL-10 on CD8+ CTL activation and tumor rejection are controversial. On one hand, IL-10 has been shown to inhibit allogeneic mixed lymphocyte reaction in human (28), to decrease monocyte activation (7), to induce local anergy to allogeneic and syngeneic tumor (29), or to protect target cells against cytolysis (30). In contrast, IL-10 increases the proliferative response of activated human CD8+ T cells (15), the frequency of CTL after polyclonal activation (31), or Ag-specific activation in vivo (32). Our results shed some light on the contradictory results obtained with IL-10 in the treatment of tumors in mouse models and suggest that IL-10 might have beneficial effects when injected after the onset of the tumor to activate cytotoxic CD8+ T cells.

The poor immune response observed after immunization of IL-10 TG mice could be due to several mechanisms. IL-10 has been shown to inhibit the Ag-presenting capability of monocytes (3) mainly by down-regulating costimulatory molecules (5, 6). However, no change in the expression of MHC molecules or CD80, CD86, or ICAM-1 was observed on macrophages from IL-10 TG mice (Table I). Moreover, no difference in the Ag-presenting capacity of purified splenic macrophage from hIL-10 TG mice was observed in vitro (not shown).

A second hypothesis to explain the absence of T cell responses after immunization of IL-10 TG mice would be the induction of anergy in T cells and the development of Tr1-type cells. Indeed, we have previously shown that activation of human CD4+ T cells in the presence of IL-10 resulted in the induction of a long-term Ag-specific anergy (10). Moreover, we have recently shown that both human and mouse CD4+ T cells, repeatedly stimulated in the presence of IL-10, differentiate into a new subset of CD4+ T cells different from the classical Th1 and Th2 T cell clones. These cells, termed Tr1 (T regulatory 1) have a poor proliferative response and secrete no IL-2 or IL-4, but do produce high levels of IL-10 and inhibit the proliferative response of bystander cells both in vitro and in vivo (11). The development of Tr1 cells in IL-10 TG mice would result in the absence of T cell-mediated response in vivo and in the absence of recall stimulation in vitro, as observed in the present study. The injection of CD4+ T cells bearing a TG OVA TCR from DO11-10 mice into IL-10 TG mice will be used to test this hypothesis.

**Acknowledgments**

We thank D. Mathis and C. Benoist for providing the Ea promotor, A. O’Garra for reviewing the manuscript, and J. Katheiser for excellent secretarial assistance.

**References**