T Cell-Derived IL-10 Promotes Lung Cancer Growth by Suppressing Both T Cell and APC Function[1]

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We have found previously that human lung cancers potently induce T lymphocyte IL-10 production in vitro. To assess the impact of enhanced T cell-derived IL-10 on antitumor immunity in vivo, we utilized transgenic mice expressing IL-10 under the control of the IL-2 promoter. We have shown previously that Lewis lung carcinoma cells (3LL) have more aggressive growth potential in IL-10 transgenic mice compared with control littermates. In this study, we show that transfer of T cells from IL-10 transgenic mice to control littermates transferred the IL-10 immunosuppressive effect and led to enhanced 3LL tumor growth. In addition to changes in T cell-mediated immunity, professional APC from IL-10 transgenic mice were found to have significantly suppressed capacity to induce MHC alloreactivity, CTL responses, and IL-12 production. Tumor Ag-pulsed dendritic cells from IL-10 transgenic mice also failed to generate antitumor reactivity. These results suggest that increased levels of T cell-derived IL-10 severely impair antitumor immunity in vivo, due to defects in both T cell and APC function. ***The Journal of Immunology,*** **1999, 163: 5020–5028.**

Although lymphocytes can infiltrate a variety of malignancies, many tumors have the capacity to evade the host's immune response (1, 2). Tumor cells may avoid lymphocyte-mediated immune responses by secreting immune inhibitory cytokines in the tumor milieu (3, 4). In addition to secreting their own suppressive mediators, tumor cells may also signal surrounding inflammatory cells to release suppressive cytokines, such as IL-10. IL-10 inhibits a broad array of immune parameters in vitro, including proinflammatory cytokine production by macrophages (5), Ag presentation (6), Ag-specific T cell proliferation (7, 8), and type 1 cytokine production by T cells (9, 10). Therefore, elevated IL-10 production at the tumor site in vivo may potently suppress Ag presentation, enabling the tumor to escape immune detection (3, 4).

We have reported previously that human lung tumor nodules produce significantly greater amounts of IL-10 than does normal lung tissue (3). Although lung tumor cells secrete IL-10, tumor cells also potently induce lymphocyte IL-10 production via a PGE_2-mediated pathway, and thus T cell-derived IL-10 appears to be the predominant source of this cytokine in the lung tumor environment (11). To assess the impact of enhanced T cell-derived IL-10 on antitumor immunity in vivo, we utilized a novel transgenic mouse model in which IL-10 is expressed under the control of the IL-2 promoter.

We have shown previously that Lewis lung carcinoma cells (3LL)[3] grow more rapidly in IL-10 transgenic mice compared with their control littermates (12). The current studies were undertaken to address the mechanisms responsible for enhanced tumor growth in the IL-10 transgenic mice. Consistent with the in vitro results, we report that IL-10 transgenic mice demonstrate a reduced capacity for Ag presentation, CTL generation, and type 1 cytokine production, reflecting defects in both T cell and APC function. Furthermore, the effects on APC persist in the absence of IL-10-producing transgenic T cells.

Materials and Methods

Cell culture

The murine Lewis lung carcinoma cell line (3LL, $H2^b$) was obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were routinely cultured as monolayers in 25-cm^2 tissue culture flasks containing RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% FBS (Gemini Bioproducts, Calabasas, CA), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 2 mM glutamine (JRH Biosciences, Lenexa, KS), and maintained at 37°C in humidified atmosphere containing 5% CO_2 in air. The cell line was mycoplasma free, and cells were utilized before the tenth passage for these studies.

Mice

IL-10 transgenic mice were made by standard methods at University of California, Los Angeles, Transgenic Mouse Core Facility (Los Angeles, CA), as previously described (12). Briefly, the human IL-2 promoter-enhancer region from −567 to +54, relative to the transcriptional start site, was cloned upstream of the mouse IL-10 genomic sequence. The mouse IL-10 sequence used in the construct corresponds to positions 1568–6879 of a 7.2-kb *Bgl*II fragment, in which position 1568 represents nucleotide 16 of the IL-10 cDNA. The construct was injected into eggs from (C57BL/6 × C3H)F_1 females mated to C57BL/6 male, and transgenic founders were

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[3] Abbreviations used in this paper: 3LL, Lewis lung carcinoma cells; TD50, tumor dose at which 50% of mice develop tumors; DC, dendritic cell; MCF, mean channel fluorescence; SEB, staphylococcal enterotoxin B.

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backcrossed onto the C57BL/6 background >5 times. Presence of the transgene was confirmed by PCR of mouse tail biopsy. The 5′ primer sequence was 5′-TGT CCA CCA CAA TAT GCT ATT CA-3′ (positions −372 to −350 of the IL-2 promoter sequence) and the 3′ primer sequence was 5′-ATA CTT ACA AAG AAA GTC TTC ACC-3′ (positions 1777–1800 of the IL-10 exon 1 of the mouse cDNA). The size of the resulting IL-10 PCR fragment is 800 bp. One microgram of DNA was amplified in a total volume of 50 μl, which contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM each dNTPs, 0.1 μM primers, 2.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase. PCR was performed in a Perkin-Elmer (Norwalk, CT) DNA thermal cycler. The amplification profile for the IL-10 transgene consisted of 40 cycles, with the first cycle denaturation at 94°C for 3 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, followed by 39 cycles with denaturation at 94°C for 1 min and the same annealing and extension conditions. The extension step in the last cycle was for 10 min. After amplification, the products were visualized against m.w. standards on a 1.5% agarose gel stained with ethidium bromide. IL-10 transgenic mice were bred at the West Los Angeles Veterans Affairs vivarium and maintained in the animal research facility. For all experiments, pathogen-free female transgenic mice or their control littermates (8–12 wk age) were used.

Tumorigenicity

For tumorigenesis experiments, 3LL tumor cells were inoculated by s.c. injection in the right supra scapular area in transgenic and control littermates, and tumor volume was monitored. Tumor growth was assessed three times per week following tumor implantation. Two bisecting diameters of each tumor were measured with calipers. The volume was calculated using the formula (0.4) (ab^2), with "*a*" as the larger diameter and "*b*" as the smaller diameter. To determine the dose of tumor-forming established nodules in 50% of mice (TD50) in transgenic and control littermates, a range of 3LL tumor cell inoculums (2.5×10^3 to 5×10^4) was injected s.c., and percentage of tumor take in the animals was recorded. To compare tumor formation in the lungs of transgenic and control littermates, 5×10^5 3LL tumor cells were inoculated i.v. via the lateral tail vein. After 34 days, mice were sacrificed and lungs were isolated for quantitation of tumor surface area. Tumor burden was assessed by microscopic examination of hematoxylin and eosin-stained sections with a calibrated graticule (a 1-cm^2 grid subdivided into one hundred 1-mm^2 squares). A grid square with tumor occupying >50% of its area was scored as positive, and the total number of positive squares was determined (13).

Lymphocyte transfer

T lymphocytes were isolated from spleens of transgenic mice by Dynal beads using the manufacturer's protocol (Dynal, Great Neck, NY). IL-10 transgenic T lymphocytes or T lymphocytes from control littermates (3.5×10^7) were transferred to each control littermate 24 h before 3LL tumor cell inoculation (5×10^5 tumor cells) and again 1 wk following tumor inoculation. Tumor volumes were assessed three times per week.

Determination of tumor nodule and splenic T cell IL-10 production

Tumor nodule-associated IL-10 protein was determined in IL-10 transgenic mice and control littermates. IL-10 protein was also determined from splenic T cells. T cells were isolated using Dynal beads coated with Thy-1.1 Ab (PharMingen, San Diego, CA), according to the manufacturer's instructions. IL-10 production was determined by cytokine-specific ELISA in T cell supernatants after 3 days in culture or directly from homogenized tumor tissue. To address whether factors derived from tumor cells could induce the IL-10 transgene, 1×10^6 splenocytes from IL-10 transgenic mice and negative littermates were cocultured with 10^5 3LL cells. Following a 3-day coculture, the IL-10 secreted in the culture supernatants was measured by IL-10 ELISA.

Flow cytometry

For flow-cytometric experiments, two or three fluorochromes (PE, FITC, and Tri-color) were used to gate on the CD11c (PharMingen) bright populations of splenic APC or bone marrow-derived dendritic cells (DC) in evaluation of MHC I, MHC II, CD40, B7.1 and B7.2, and F480 (Caltag, San Diego, CA). Flow-cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) in the University of California, Los Angeles, Jonsson Cancer Center Flow Cytometry Core Facility. Cells were identified as APC by gating based on forward and side scatter profiles. Between 3000 and 5000 gated events were collected and analyzed using CellQuest software (Becton Dickinson).

To determine the source of enhanced IL-10 in the tumor nodules of IL-10 transgenic mice, tumors were harvested, cut into small pieces, and passed through a sieve (Bellco, Vineland, NJ). Intracytoplasmic staining for IL-10 with a PE-labeled anti- mouse IL-10 mAb (PharMingen) and cell surface staining for CD3 with FITC-labeled anti-mouse CD3 were performed, followed by flow cytometry. Tumor cells were identified by gating on in vitro cultured 3LL cells.

Mixed lymphocyte reaction

To compare the T cell stimulatory capacity of APC from transgenic mice and the control littermates, MLR were performed utilizing BALB/c ($H\text{-}2^d$) T cells as the responder cells. Splenic APC ($H\text{-}2^b$) from transgenic and control littermates were purified utilizing Ab-mediated complement lysis of T and B cell populations. The following Abs, all obtained from ATCC, were utilized: TIB 207 (anti-CD4), TIB 150 (anti-CD8), TIB 146 (anti-B lymphocyte). After RBC depletion, splenocytes were incubated with a mixture of mAbs and rabbit complement for 60 min at 37°C. Following Ab-mediated depletion, APC were washed twice in complete medium. Subsequently, T cells were purified from BALB/c spleens utilizing Ab-mediated complement lysis of APC and B lymphocytes (Abs TIB 229 and TIB 146; ATCC). APC from transgenic mice and control littermates were co-cultured with splenic BALB/c T cells at varying APC:T cell ratios in 96-well tissue culture plates for 5 days. The responder cell numbers were kept constant and APC numbers were varied. On day 4, the cultures were pulsed with 1 μCi of tritiated thymidine (Amersham, Arlington, IL; sp. act., 62 Ci/mmol/L) for 18 h, and the cells were harvested onto filter mats using a Skatron Cell Harvester. The disks were air dried, placed in scintillation vials with 1 ml of scintillation fluid, and counted in a Beckman scintillation counter (Fullerton, CA). To assess T cell reactivity in IL-10 transgenic and control littermates, MLR were performed with APC from BALB/c ($H\text{-}2^d$) mice as stimulators. MLR were also set up with day 8 matured bone marrow-derived DC from IL-10 transgenic mice and control littermates as stimulators with T cells from BALB/c mice as responders.

APC IL-12 production

APC were purified from total splenocyte suspension by Ab-mediated complement lysis of T and B cells, as described above. APC (5×10^6 cells/ml) from tumor-bearing transgenic mice and control littermates were stimulated with or without anti-CD40 (5 μg/ml). Following a 72-h culture, IL-12 secreted in culture supernatants was determined by ELISA.

Cytokine ELISA

IL-10 protein concentrations from mouse splenocytes cultured in complete medium or 3LL cell supernatants were determined by IL-10-specific ELISA, as previously described (3). Briefly, 96-well Costar (Cambridge, MA) plates were coated overnight with 4 μg/ml of anti-mouse IL-10 mAb JES5-2A5 (PharMingen). The wells of the plate were blocked with 10% FBS (Gemini Bioproducts, Calabasas, CA) in PBS for 30 min. The plate was then incubated with the Ag for 1 h and excess Ag was washed off with PBS/Tween. The plate was incubated with 1 μg/ml of biotinylated mAb to IL-10 (PharMingen) for 30 min, and excess Ab was washed off with PBS/Tween. The plates were incubated with avidin peroxidase, and after incubation in *o*-phenylenediamine substrate, the subsequent change in color was read at 490 nm with a Dynatech MR5000 spectrophotometer (Chantilly, VA). The rIL-10 used in the assay as a standard was obtained from PharMingen. For IL-2 and IFN-γ determinations, the above procedure was repeated, but with either IL-2 or IFN-γ Ab pairs and standards from PharMingen. The sensitivity of the IL-10, IL-2, and IFN-γ ELISA was 15 pg/ml. For IL-12 measurements, an IL-12 ELISA kit was utilized (Biosource International, Camarillo, CA) and measurements were performed according to the manufacturer's instructions. The sensitivity of the IL-12 ELISA was 5 pg/ml.

Assessment of type 1 cytokine production in vivo

To evaluate Th1 cytokine production in vivo, IL-10 transgenic mice and control littermates were injected i.v. with staphylococcal enterotoxin B (SEB; 50 μg) dissolved in pyrogen-free NaCl (0.9%). The mice were bled 2 h later, and the sera were assayed for IL-2 and IFN-γ production by ELISA.

Assessment of CTL activity

To evaluate the function of CTL from IL-10 transgenic and control littermates, mice were vaccinated three times at weekly intervals with irradiated 5×10^6 3LL cells. Splenic lymphocyte cytotoxicity was determined against 3LL tumors, as previously described. Briefly, 5×10^6/ml (5 ml total) RBC-depleted splenocytes from IL-10 transgenic and control littermates were stimulated in the presence of 5×10^5 mitomycin C-treated 3LL cells and IL-2 (100 U/ml) for 10 days with one medium change on day 5.

CTL cytotoxicity was determined against chromium-labeled (^{51}Cr; sp. act., 250–500 mCi/mg; Amersham) 3LL targets at varying E:T ratios for 4 h in 96-well plates. Spontaneous release and maximum release with 5% Triton X also were assessed. Following the 4-h incubation, supernatants were removed and activity was determined using a gamma counter (Beckman). The percent specific lysis was calculated using the formula: percentage lysis = 100 × [(experimental cpm − spontaneous release)/(maximum release − spontaneous release)].

Isolation, in vitro propagation, and peptide loading of DC

Lymphocyte-depleted mouse bone marrow cells were cultured with murine GM-CSF (2 ng/ml) and IL-4 (20 ng/ml) (R&D Systems, Minneapolis, MN) for 8 days, as previously reported (14). Medium was replenished every other day. On day 8, nonadherent DC were isolated by pipetting. The DC were counted and washed twice in PBS, and 1×10^6 DC were prepared for loading with peptides. The MUT 1 and MUT 2 peptides, synthesized by Research Genetics (Huntsville, AL), consist of the 52–59 aa positions of the mutated connexin 37 protein present in the 3LL cell line and have the sequence FEQNTAQP and FEQNTAQA, respectively. Bone marrow-derived DC were incubated in 1 ml of RPMI with 10 μM peptide for 2 h at 37°C. Following incubation, the peptide-loaded DC were washed twice in PBS, and 1×10^6 peptide-loaded DC were administered by intratumoral injection at weekly intervals for 3 weeks in mice bearing 5-day-old established s.c. tumors that were generated previously by implanting 10^5 tumor cells.

Results

3LL tumorigenicity is augmented in IL-10 transgenic mice

Our previous studies documented that 3LL tumors cells grew more rapidly in IL-10 transgenic mice than did tumors in control mice. This effect was reversed by in vivo administration of anti-IL-10 mAb (12). To further assess tumorigenicity of 3LL cells in IL-10 transgenic mice, mice were challenged with a range of tumor cell inoculums (2.5×10^3 to 5×10^4 cells) and the percentage of mice with tumors and tumor volumes were monitored. When compared with littermate controls, the 3LL tumor cells were significantly more tumorigenic in IL-10 transgenic mice up to inoculums of 2.5×10^4 tumor cells. The TD50 in IL-10 transgenic mice was significantly less than the TD50 in controls (7.5×10^3 vs 2.2×10^4 cells, respectively, $p < 0.01$, Fig. 1*A*). Because different organ sites may have varying capacities to support or limit tumor propagation (15), the growth of 3LL was assessed in the lungs of IL-10 transgenic mice following i.v. tumor challenge. Thirty-four days following tumor challenge, IL-10 transgenic mice had a significantly greater tumor burden within the lungs than did controls ($p < 0.05$, Fig. 1*B*). Thus, the IL-10 transgenic mice demonstrated a diminished capacity to limit tumor growth in both s.c. and pulmonary sites.

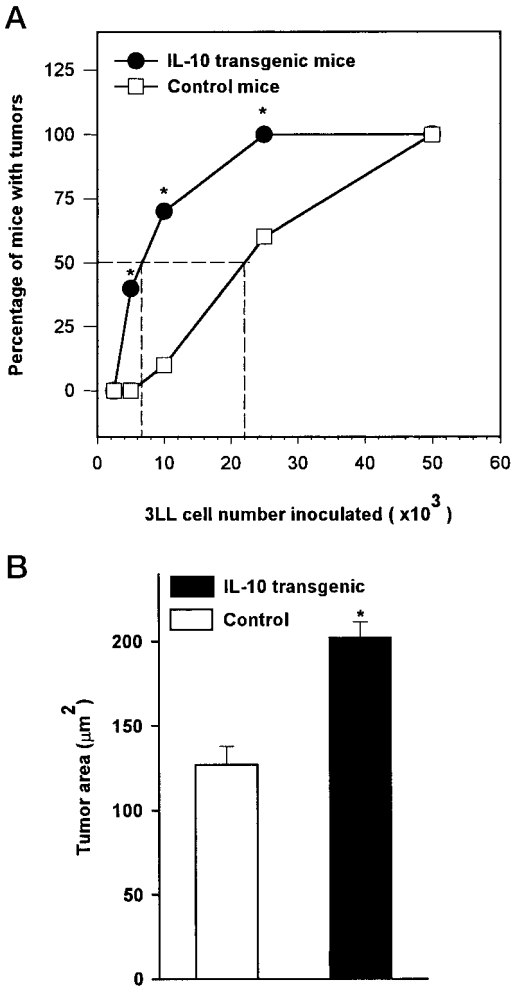

FIGURE 1. Tumorigenicity of 3LL is augmented in IL-10 transgenic mice. *A*, The TD50s in IL-10 transgenic and control C57BL/6 mice were determined by injecting 3LL cells s.c. in inoculums ranging from 2.5×10^3 to 5×10^4 cells. The percentages of established tumor nodules in each group of mice were recorded. *B*, Experimental pulmonary metastases show enhanced growth within the lungs of IL-10 transgenic mice (*, $p < 0.05$). Five hundred thousand 3LL cells were administered i.v. via a lateral tail vein in transgenic and control mice. After 34 days, lungs were isolated and paraffin-embedded sections were prepared for H&E staining. Tumor area was quantified within the lung by microscopy, as described in *Materials and Methods* (n = 10–12 mice per group per dose of cells utilized).

Enhanced local and systemic IL-10 production in tumor-bearing IL-10 transgenic mice

Based on the findings noted above, we speculated that IL-10 production would be elevated in IL-10 transgenic mice both locally, at the tumor site, and systemically, within the spleen. To confirm that the IL-10 transgene was functional in the IL-10 transgenic mice, splenocyte IL-10 production was assessed in cells from transgenic and control mice using in vitro stimulation. There were no significant differences in constitutive IL-10 production between the groups. In agreement with previous results (12), stimulation of freshly isolated spleen cells with one of several stimuli, including anti-CD3 mAb, IL-2, or Con A, led to significantly more IL-10 production in cells from IL-10 transgenic mice (data not shown). To determine the extent of splenic IL-10 production in vivo, within the context of the tumor model, IL-10 production by splenic T cells from IL-10 transgenic mice and control littermates bearing 3LL tumors was determined following a 3-day culture (Table I). In nontumor bearers, unstimulated splenic T cells from IL-10 transgenic mice produced 354 ± 40 pg/ml of IL-10 following in vitro culture, whereas unstimulated splenic T cells from control mice did not produce detectable IL-10. In 3LL tumor bearers, splenic T cells from the control mice produced detectable IL-10, but splenic T cells from IL-10 transgenic mice produced significantly more of this cytokine than did splenic T cells from the control mice (Table I). Following culture, unstimulated splenic T cells from transgenic

Table I. *IL-10 transgenic splenic T cells show enhanced IL-10 production*[a]

Splenic T cells	IL-10 (pg/ml)	
	Control mice	IL-10 transgenic mice
Nontumor bearer	0	354 ± 40
Tumor bearer	652.7 ± 24.7	1094.2 ± 65

[a] T cells were isolated by positive selection using Thy 1.1-coated Dynal beads. Splenic T cells (2×10^6/ml) from IL-10 transgenic and control mice were incubated in culture medium for 3 days, and IL-10 secreted in the supernatants was measured by IL-10-specific ELISA. Results are representative of three individual experiments.

Table II. *Tumor nodule IL-10 concentrations are significantly elevated in IL-10 transgenic mice*[a]

Source	IL-10 (pg/gm of tissue/ml)	
	Control mice	IL-10 transgenic mice
3LL tumor nodule	608 ± 24	14830 ± 75

[a] Non-necrotic tumors from IL-10 transgenic and control mice were homogenized and IL-10 concentrations were determined by specific ELISA. (n = 5 mice/group). Results are representative of three separate experiments.

mice made significantly more IL-10 whether or not mice had been injected with tumor. The relative increase in IL-10 production in the tumor-injected animals vs the nontumor-bearing mice is similar for both nontransgenic and IL-10 transgenic mice. To address whether factors derived from tumor cells could induce the IL-10 transgene, splenocytes from IL-10 transgenic mice and negative littermates were cocultured with 3LL cells. The IL-10 secreted in the culture supernatants was measured by IL-10 ELISA. Following a coculture with 3LL cells, there was an enhanced IL-10 production from IL-10 transgenic splenocytes (14,210 ± 36 pg/ml) compared with splenocytes from negative littermates (3,461 ± 120 pg/ml). The 3LL tumor cells and unstimulated splenocytes from transgenic and negative littermates produced only modest amounts (160 ± 14 pg/ml, 160 ± 10 pg/ml, 173 ± 9 pg/ml, respectively). The enhanced production of lymphocyte-derived IL-10 in normal tumor-bearing mice has been previously described (16) and may be related to PGE_2-mediated stimulation of lymphocyte IL-10 production (17). The effect of the IL-10 transgene on local IL-10 production was even more striking. Tumor nodules from IL-10 transgenic and control mice were analyzed directly for IL-10 following homogenization. Tumor nodules from IL-10 transgenic mice produced significantly more IL-10 than did tumor nodules from control mice (Table II). To determine the source of the enhanced IL-10 in the tumor nodules of IL-10 transgenic mice compared with tumor nodules from negative littermates, intracytoplasmic staining for IL-10 and cell surface staining for CD3 followed by flow cytometry were performed. The results of these experiments show that the predominant source of IL-10 in the tumor nodules from IL-10 transgenic mice are T cells with a very small contribution from tumor cells. Within the gated lymphocyte population, 50% of cells stained positively for both CD3 and intracytoplasmic IL-10 in single cell suspensions of the tumor nodule from IL-10 transgenic mice. In contrast, only 0.4% of 3LL tumor cells stained positively for intracytoplasmic IL-10. Within the gated lymphocyte population, CD3 cell surface staining for T cells showed that the tumor nodules in IL-10 transgenic mice had 3 times more T lymphocytes than tumor nodules of negative littermates (60 vs 20%, respectively, $p < 0.01$).

Limited type 1 cytokine induction in IL-10 transgenic mice

In both patients and murine tumor models, progressive tumor growth has often been associated with a marked limitation in lymphocyte Th1 cytokine production along with up-regulated IL-10 and Th2 cytokines (16, 18–21). We hypothesized that, similar to tumor bearers, lymphocytes from IL-10 transgenic mice would have a decrement in their capacity to elaborate IL-2 and IFN-γ in vivo (22). Administration of the superantigen SEB to normal mice rapidly elicits the production of the Th1 cytokines IL-2 and IFN-γ (23). This is a potent and consistent stimulus for Th1 cytokine production in vitro and in vivo, with the early cytokine production derived from memory and NK T cells. To assess the level of induction of IL-2 and IFN-γ in vivo in IL-10 transgenic and control mice, SEB was administered i.v. in mice with or without 3LL tumors. After 2 h, the mice were bled and the sera assayed for IL-2 and IFN-γ by ELISA. Following SEB injection, IL-10 transgenic mice produced significantly less IFN-γ (120 ± 15 vs 1250 ± 50 pg/ml, $p < 0.05$) and IL-2 (265 ± 20 vs 670 ± 100 pg/ml, $p < 0.05$) than the control SEB-injected mice (Fig. 2, *A* and *B*). Control mice bearing 3LL tumors had a reduction in IL-2 and IFN-γ production in response to SEB. In response to SEB, nontumor-bearing IL-10 transgenic mice produced similar levels of IL-2 and IFN-γ as did control mice bearing tumors (Fig. 2, *A* and *B*). Tumor-bearing IL-10 mice produced even lower levels of IFN-γ and IL-2 than did their nontumor-bearing, IL-10 transgenic counterparts.

CTL from transgenic mice have reduced capacity to lyse 3LL tumors

CTL induction may be viewed as a hallmark of effective antitumor reactivity. To assess CTL activity, IL-10 transgenic mice and

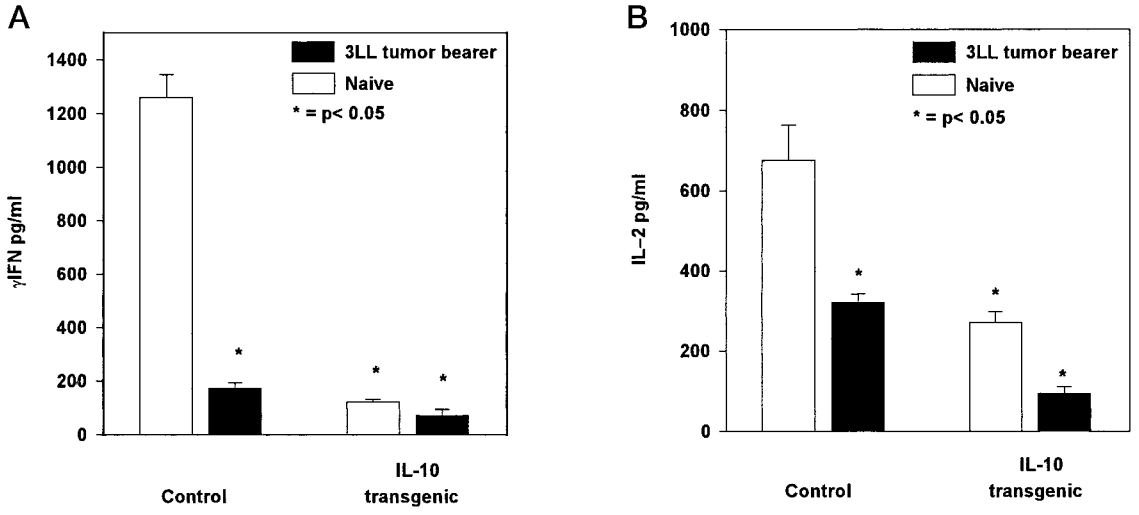

FIGURE 2. IL-10 transgenic mice have a diminished capacity to generate Th1 cytokines in response to superantigens. SEB (50 μg/mouse) was administered i.v. via a lateral tail vein to IL-10 transgenic and control mice. The mice were bled 2 h later, and the sera were assayed for IL-2 and IFN-γ by cytokine-specific ELISA (*, $p < 0.05$, compared with control mice without tumor). In IL-10 transgenic mice, there was a significant reduction in the production of IFN-γ (*A*) and IL-2 (*B*) in response to SEB ($n = 6$ mice/group).

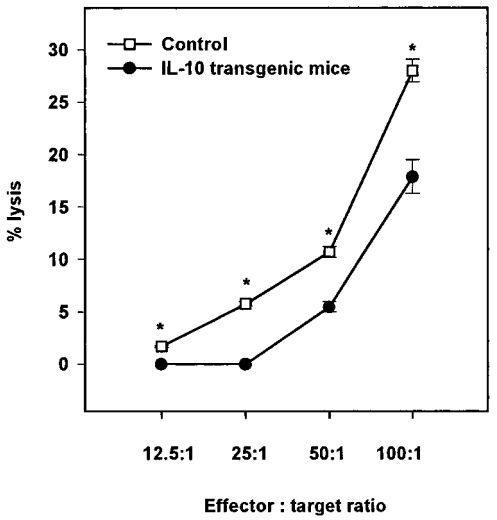

FIGURE 3. CTL activity is reduced in IL-10 transgenic mice. CTL cytotoxicity against parental 3LL tumor cells was assessed in IL-10 transgenic and control mice. CTL against 3LL cells were generated by vaccinating IL-10 transgenic and control mice with irradiated 3LL cells. Following vaccination, splenocytes were isolated and restimulated in vitro with irradiated 3LL cells, and CTL cytotoxicity was assessed in chromium release assays. Results are representative of three assays (*, $p < 0.05$, compared with control).

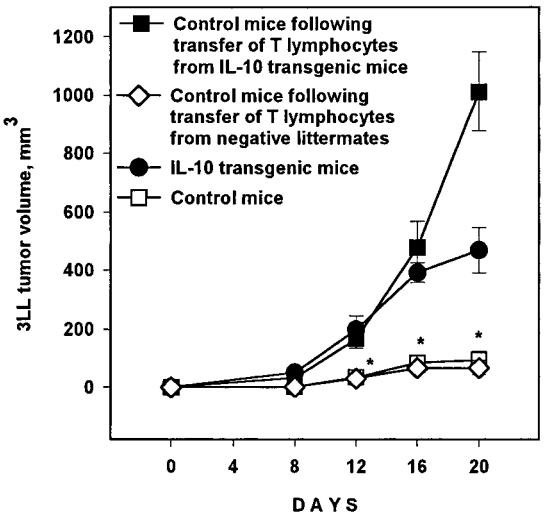

FIGURE 4. Intravenous transfer of IL-10 transgenic splenic T cells to normal mice reduces the capacity to limit tumor growth. Splenic T lymphocytes from IL-10 transgenic mice (3.5×10^7 cells) or control littermates were transferred to control littermates 24 h before and 1 wk following tumor challenge. Tumor volumes were assessed three times each week. Following transfer of IL-10 transgenic T cells, the growth of 3LL in normal mice was significantly enhanced and approximated the rate of growth of tumors in IL-10 transgenic mice (*, $p < 0.05$, compared with controls) ($n = 6$ mice/group).

control littermates were vaccinated three times at weekly intervals with irradiated 3LL cells. Following vaccination, spleens were isolated and splenic lymphocytes were restimulated with mitomycin C-treated 3LL cells. CTL from IL-10 transgenic and control mice were evaluated for cytolytic activity against parental 3LL tumors. CTL from IL-10 transgenic mice were less effective than were CTL from control mice in lysing 3LL targets (Fig. 3).

IL-10 transgenic T cells transfer the immunosuppressive effects

Previous studies suggest that populations of T cells in the tumor-bearing host may develop suppressor activities through the induction of IL-10 gene expression (11, 16). We therefore predicted that adoptive transfer IL-10 transgenic lymphocytes to control littermates would diminish the capacity of the recipients to limit 3LL growth in vivo. To determine whether the immunosuppressive effect could be transferred, 3.5×10^7 IL-10 transgenic splenic T cells were administered to control mice 1 day before and 1 wk following tumor challenge. Following i.v. transfer of IL-10 transgenic T cells to control mice, the 3LL tumor growth revealed a similar pattern to that demonstrated in IL-10 transgenic mice (Fig. 4), whereas transfer of T cells from negative littermates to control mice did not alter the growth pattern of 3LL tumors. Thus, lymphocytes from IL-10 transgenic mice demonstrated a dominant immunosuppressive effect in control animals, limiting antitumor responses in normal hosts following transfer.

APC from IL-10 transgenic mice fail to support MLR

Although IL-10 has been shown to directly inhibit lymphocyte activity (24), studies indicate that the predominant pathway for inhibition of lymphocyte effector functions most often depends upon the capacity of IL-10 to modify APC surface phenotype and cytokine production (5, 10, 25, 26). We therefore evaluated a range of APC activities, including the promotion of MHC alloreactivity, CTL induction, cytokine production, and the induction of antitumor reactivity in vivo.

IL-10 has previously been documented to potently inhibit the proliferation and cytokine production of alloreactive T cells in MLR (27). We postulated that exposure to lymphocyte-derived IL-10 in vivo would lead to a reduction in the capacity of APC to promote MLR. Utilizing BALB/c T cells as the responder cells, mixed lymphocyte reactions were performed to compare the T cell stimulatory capacity of APC from transgenic mice and the control littermates. In comparison with cells from control mice, both splenic APC and bone marrow-derived DC from IL-10 transgenic mice revealed a marked reduction in the capacity to generate an MLR with allogeneic BALB/c responder T cells ($p < 0.01$) (Figs. 5*A* and 6). Consistent with these findings, when assessed by flow cytometry, splenic APC from IL-10 transgenic mice had a marked reduction in the mean channel fluorescence (MCF) intensity of B7.1, B7.2, MHC I, and CD11c expression ($p < 0.05$) (Table III). In contrast, when MLR was performed with splenic T lymphocytes from IL-10 transgenic mice with normal BALB/c APC, there was no significant difference in the proliferative response compared with the control MLR with normal lymphocytes (Fig. 5*B*). The inability to detect a difference in T cells in this assay may reflect the subtle alteration in cytokine pattern in naive T cells from IL-10 transgenic mice.

As described above, splenic APC obtained directly from IL-10 transgenic mice revealed a reduced capacity to generate MLR. DC, the most potent APC, are capable of effectively stimulating naive T cells (28, 29). Because DC may be central to the induction of the antitumor immune response and IL-10 has previously been demonstrated to modulate their maturation, phenotype, function, and access to the tumor site (6, 30–34), we assessed bone marrow-derived DC from transgenic mice for their capacity to generate MLR and mediate antitumor effects in vivo. Bone marrow-derived DC were generated from IL-10 transgenic and control mice by in vitro culture in GM-CSF and IL-4. Similar to the results found with splenic APC, bone marrow-derived DC from IL-10 transgenic mice showed a significant reduction in MLR reactivity compared with DC from control mice (Fig. 6). Consistent with these findings, DC from IL-10 transgenic mice evidenced a significant reduction in the expression of B7.1 and MHC II expression (Table IV).

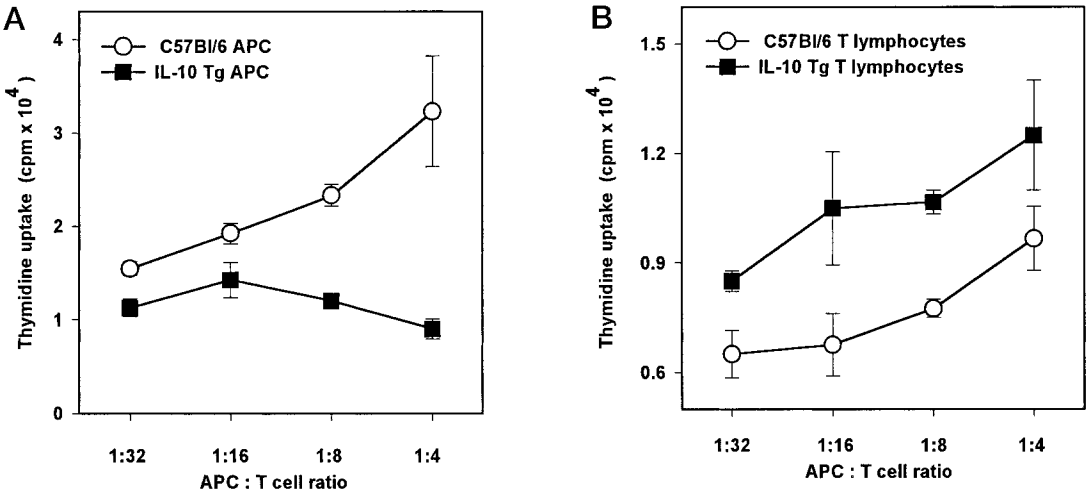

FIGURE 5. *A*, Splenic APC from IL-10 transgenic mice reveal a reduced capacity to generate alloreactivity. MLR with APC from IL-10 transgenic or control littermates were performed with responder T lymphocytes from BALB/c mice. The responder cell numbers were kept constant and the APC numbers varied. APC from IL-10 transgenic mice exhibited a reduced MLR compared with the control mice (*, $p < 0.05$). *B*, Splenic T cells from IL-10 transgenic mice generate normal levels of alloreactivity in MLR with BALB/c APC (*, $p < 0.05$). Results are representative of three separate experiments.

DC from IL-10 transgenic mice fail to induce antitumor reactivity

To evaluate the capacity of DC to mediate specific in vivo antitumor effects, the in vitro activated DC were loaded with MUT 1 and MUT 2 peptides, and their capacity to cure 5-day-old established tumors in IL-10 transgenic and control mice was assessed. C57BL/6 DC loaded with MUT 1/MUT 2 peptides mediated complete eradication of established tumors in all normal control mice, but it mediated eradication in only two of six animals when treating IL-10 transgenic mice bearing 3LL tumors. DC from IL-10 transgenic mice loaded with MUT 1/MUT 2 peptides were ineffective in mediating any significant tumor reduction in C57BL/6 control mice bearing 3LL, thereby demonstrating APC as well as T cell defects (Table V). These APC defects were apparent even after short-term (8-day) culture in the absence of T cells. To determine whether contaminating lymphocytes producing IL-10 within our DC cultures were contributing to modifications in function and phenotype, we repeated these experiments using anti-IL-10 mAb within the medium throughout the culture period. No restoration of DC function was observed, suggesting that the detrimental exposure to IL-10 occurred in vivo (data not shown).

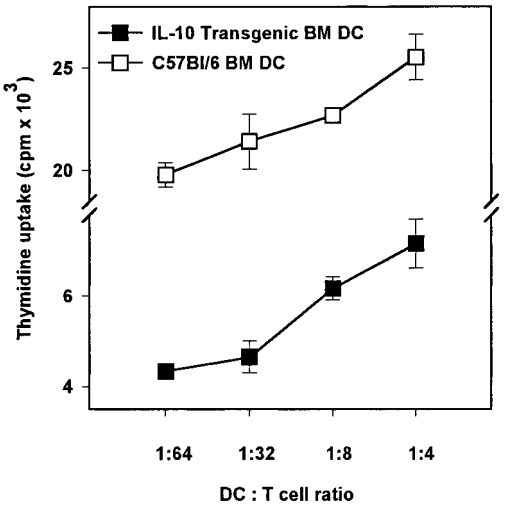

FIGURE 6. DC from IL-10 transgenic mice reveal a reduced capacity to generate alloreactivity. IL-10 transgenic and control bone marrow-derived DC propagated in GM-CSF and IL-4 for 8 days were assessed in MLR with BALB/c T lymphocytes as responders. The responder cell numbers were kept constant and the APC numbers varied. Following a 3-day culture, T cell proliferation was determined by ^{3}H uptake (*, $p < 0.05$). Results are representative of three separate experiments.

APC from IL-10 transgenic mice have limited capacity to produce IL-12

The capacity for professional APC to produce IL-12 has been correlated with APC functional maturation (35). To determine the role of increased lymphocyte-derived IL-10 on splenic APC IL-12 production, IL-12 released by IL-10 transgenic and control mice splenic APC was assessed. Unstimulated APC from control mice, prepared by negative selection of T and B cells, produced significantly more IL-12 than did APC from IL-10 transgenic mice (110 ± 4.5 vs 25 ± 2.5 pg/ml, respectively; $p < 0.01$). Following stimulation with anti-CD40 mAb, APC from control mice produced a 3.5-fold greater induction of IL-12 than did the APC from IL-10 transgenic mice (Fig. 7). To determine whether the reduced levels of IL-12 production from APC in IL-10 transgenic mice were accompanied by a decrement in CD40 receptor expression by IL-10 transgenic APC, flow-cytometric analysis was performed. APC from IL-10 transgenic mice expressed significantly less cell surface CD40 than did controls (28% (233 MCF) vs 64% (458

Table III. *Splenic APC from IL-10 transgenic mice show modified surface phenotype*[a]

Surface Ag	% Positive (MCF intensities)	
	Control APC	IL-10 transgenic APC
CD11c	23 (332)	21 (180)*
CD40	64 (458)	28 (233)*
B7.1	25 (1420)	27 (1144)*
B7.2	69 (2271)	58 (1127)*
MHC-I	99 (3468)	97 (2029)*
MHC-II	35 (753)	23 (721)
F480	31 (596)	34 (507)

[a] IL-10 transgenic and control mice APC were analyzed by flow cytometry. The table depicts % positively stained cells and MCF intensities (*, $p < 0.05$). Results are representative of three separate experiments.

Table IV. *Bone marrow-derived DC from IL-10 transgenic mice show modification of surface phenotype*[a]

Marker	% Positive (MCF intensities)	
	C57B1/6 DC	IL-10 transgenic DC
CD11c	90 (511)	80 (176)*
MHC-I	73 (538)	79 (530)
MHC-II	67 (1372)	80 (1033)*
B7.1	89 (1209)	62 (667)*
B7.2	51 (650)	53 (731)

[a] Flow cytometric analysis of IL-10 transgenic and negative control mice DC following propagation in GM-CSF and IL-4 for 8 days. The numbers depict percentage of positively stained cells and mean channel fluorescence intensities (*, $p < 0.05$). Results are representative of three separate experiments.

MCF), $p < 0.05$) (Table III). Further studies will be required to determine whether the decreased expression of CD40 is the cause of limited IL-12 production.

Discussion

IL-10 is a type 2 cytokine that inhibits a broad array of immune parameters, including proinflammatory cytokine production by macrophages (5), Ag presentation (6, 10), Ag-specific T cell proliferation (7, 8), and type 1 cytokine production (5, 9, 10). Because it can accelerate DC apoptosis (36), IL-10 may be profoundly detrimental to the capacity of the host to initiate and maintain antitumor reactivity. We have reported previously that human lung cancers potently induce lymphocyte IL-10 production (11). To assess the role of enhanced T cell-derived IL-10 in the suppression of antitumor immunity in vivo, we utilized transgenic mice expressing IL-10 under the control of the IL-2 promoter. We have shown that 3LL tumor cells are less immunogenic and have a more aggressive growth potential in transgenic mice compared with control littermates (12). These changes in the capacity to limit tumor growth are IL-10 dependent, as evidenced by the restoration of antitumor responses in the transgenic mice following administration of anti-IL-10 mAb (12). In this study, we have confirmed these results, demonstrating that s.c. 3LL challenge in IL-10 transgenic mice reveals a significant reduction in TD50. Furthermore, this immune deficit was not limited to s.c. tumor, as i.v. challenge also led to an increased pulmonary tumor burden in IL-10 transgenic mice. Because rIL-10 had no direct effect on 3LL tumor cell proliferation in vitro, we chose to delineate potential immune-mediated regulatory pathways controlled by IL-10 in vivo. IL-10 remained elevated within the context of the in vivo tumor model, as evidenced by the fact that tumor nodules from transgenic mice contained significantly more IL-10 than did tumor nodules from control littermates. To understand the immunological significance of the enhanced lymphocyte-derived IL-10 in transgenic mice, T cell and APC functions were assessed in both tumor-bearing and nontumor-bearing transgenic and control mice.

Table V. *Bone marrow derived DC from IL-10 transgenic mice have a reduced capacity to eradicate established tumors*[a]

Group	Complete Tumor Eradication/Total Treated	
	Transgenic mice	Control littermates
IL-10 DC MUT 1 MUT 2	1/6	0/6
C57 DC MUT 1 MUT 2	2/6	7/7

[a] Mice were injected s.c. with 10^5 3LL tumor cells. Bone marrow-derived DC from IL-10 transgenic mice and control littermates were activated in vitro with IL-4 and GM-CSF for 8 days. The in vitro-activated DC were loaded with MUT 1 and MUT 2 peptides and their capacity to eradicate 5-day-old established s.c. tumors in IL-10 transgenic and control mice was assessed. (n = 6–7 mice/group).

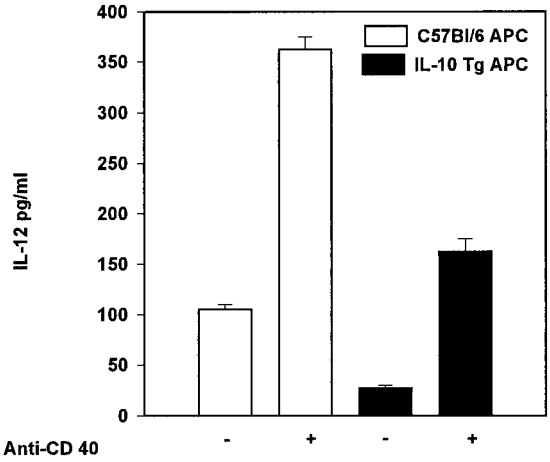

FIGURE 7. APC from IL-10 transgenic mice have a reduced capacity to secrete IL-12 both constitutively and in response to anti-CD40 mAb. Splenic APC (5×10^6 cells/ml) from IL-10 transgenic and control mice were incubated in culture medium with or without anti-CD40 mAb (5 μg/ml) for 3 days. IL-12 secreted in the supernatants was quantified by cytokine-specific IL-12 ELISA (*, $p < 0.05$). Results are representative of three separate experiments.

In the tumor-bearing host, a decrement in type 1 cytokine production has been suggested to play a significant role in the insufficient induction of specific cell-mediated antitumor immunity (22, 37). Based on our previous studies (11, 12, 38) and other recent reports (16, 39, 40), we postulated that lymphocyte-derived IL-10 could contribute significantly to reduced type 1 cytokine production capacity in vivo. To assess this parameter, we administered a potent type 1 cytokine inducer to IL-10 transgenic and control mice. Superantigens, including SEB, have been demonstrated to bind APC MHC class II molecules and stimulate a large population of $CD4^+$ and $CD8^+$ lymphocytes through interactions with specific TCR Vβ phenotypes (23). As a result of these interactions, IL-2 and IFN-γ, as well as other cytokines, are potently induced. Our findings of reduced IL-2 and IFN-γ release in IL-10 transgenic mice in response to SEB are consistent with the results of Florquin et al. (41) and Bean et al. (42). These studies demonstrated that IL-10 limits the lethal effects of SEB challenge in vivo. Thus, the readily induced, augmented levels of IL-10 produced by lymphocytes in IL-10 transgenic mice may directly limit their capacity for IL-2 and IFN-γ production.

Although IL-10 has been shown to have the capacity to directly suppress T cell activities (24), the inhibitory effects of IL-10 have most often been found to be mediated indirectly through modulation of APC function (5, 10, 25, 26). IL-10 has the capacity to decrease APC MHC and TAP expression and to down-regulate critical costimulatory molecules including CD54, CD80, and CD86 (26, 43–45). Based on these previously documented activities of IL-10, a range of immune parameters was evaluated in the IL-10 transgenic mice, including APC functional activities and phenotype, cytokine production, alloreactivity, CTL generation, and the capacity for DC to mediate therapeutic antitumor reactivity.

An important in vitro correlate of graft or tumor rejection, the MLR is able to discriminate lymphocyte activation that occurs as a function of disparate MHC expression. IL-10 has previously been reported to potently decrease the capacity of alloreactive cells

to proliferate in MLR (27). Our analysis of MLR, with IL-10 transgenic mice-derived APC serving as stimulators, revealed a significant decrease in the proliferative response of normal BALB/c lymphocyte responder cells. In contrast, normal levels of MHC alloreactive proliferation were seen when IL-10 transgenic T cells were used as responders in MLR with normal BALB/c APC. This indicates APC obtained from the IL-10 transgenic mice have an impaired capacity to generate alloreactivity, but that the transgenic T lymphocyte responses remain relatively intact. Thus, although injection of IL-10 transgenic lymphocytes transferred the immune deficit to normal mice, these results suggest that the deficit mediating immune suppression may be predominantly related to lymphocyte-induced modification of APC function and phenotype. In accordance with these findings, APC surface phenotype in IL-10 transgenic mice differed significantly from the phenotype in control mice. We found a significant decrement in surface expression of CD11c, CD40, B7-1, B7-2, and MHC class I and II.

A decreased capacity for Ag presentation by DC has been suggested to be a critical problem responsible for limitations in tumor immunity (46). To overcome this problem, investigators are using the administration of gene-modified or Ag-pulsed, in vitro activated DC to treat tumors in vivo (47–49). Although specific peptide Ag-pulsed DC from normal mice were highly effective in treating established tumors in control mice, bone marrow-derived DC from IL-10 transgenic mice were ineffective in mediating antitumor responses. This suggests that the exposure to T cell-derived IL-10 in vivo limited the capacity of precursors to undergo functional maturation despite culture in optimal concentrations of IL-4 and GM-CSF in vitro even in the presence of blocking IL-10 mAb. In accordance with these findings, the in vitro generated DC from the IL-10 transgenic mice revealed diminished surface expression of the costimulatory and MHC molecules known to be requisite for fully functional DC Ag presentation. In contrast to our studies evaluating prolonged in vivo exposure of bone marrow-derived DC, DeSmedt et al. (50) found that short-term IL-10 exposure in vitro did not alter surface phenotype. In parallel experiments, we also assessed the capacity for Ag-pulsed DC derived from normal control mice to restore antitumor reactivity in IL-10 transgenic mice. Despite this intervention, the majority of mice succumbed to progressive tumor growth, indicating a profound suppression in the capacity to react to optimal Ag presentation in vivo.

Previous studies have suggested pathways whereby IL-10 may limit antitumor responses. Pretreatment with IL-10 protects target cells from lysis by tumor-specific cytotoxic T cells (51, 52). IL-10 has been found to be released by lymphocytes infiltrating several different tumors, including ovarian (53), lung (11, 20), and cutaneous carcinomas (21). Elevated concentrations of IL-10 within the tumor environment have been suggested to limit access of DC to the tumor site (30) and to promote DC apoptosis (36). We have found IL-10 production in cutaneous basal and squamous carcinomas and documented that this cytokine provides a mechanism for evasion of the local T cell-mediated immune response (4). In a mouse model, Wang et al. (54) demonstrated that tumor cells transfected with the IL-10 gene produce local immunosuppression and prevent the induction of CTL. These results suggest that the reported effects of IL-10 on in vitro assays of Ag presentation may correlate with its effects in situ in the tumor environment. However, the literature also contains reports from studies in murine models suggesting that high local concentration of IL-10 from IL-10-transfected tumors leads to inhibition of tumor growth (55–57). These findings appear to be consistent with the fact that IL-10 has also been shown to be a positive factor for T cell differentiation, chemoattraction, and costimulation, and is a survival factor for IL-2-deprived T cells (59–63) The apparent contradiction may be explained by differences in IL-10-mediated outcomes due to concentration-dependent effects. For example, the report from Giovarelli et al. (55) showed IL-10-mediated tumor reduction in transfected murine tumors producing 2000 ng/ml of IL-10/10^6 cells/48 h. These pharmacologic cytokine levels are not comparable with physiologic concentrations, and thus may be expected to have different effects. In normal mice-bearing 3LL, we find that tumors produce 0.6 ± 0.024 ng/ml of IL-10 per gram of tumor tissue. In contrast to studies such as Giovarelli et al. (55), in our IL-10 transgenic mouse model, we find that 3LL tumor nodules produce 14.8 ± 0.075 ng/ml of IL-10 per gram of tumor tissue. Thus, while recent studies indicate that pharmacological levels of rIL-10 or high level production of IL-10 from transduced tumors promote tumor regression, our results indicate that physiologic levels of lymphocyte-derived IL-10 inhibit tumor immunity. Thus, as suggested by Suzuki et al. (56), when IL-10 is expressed at very high local concentrations in transfected tumors, the stimulatory effects on T cells may obscure the immunosuppressive effects on Ag-presenting and accessory cell functions of DC and macrophages. As recently reported by Groux et al. (58), a similar T cell stimulatory effect may be evident in a transgenic model in which APC overproduce IL-10. In their study, IL-10 caused a biphasic effect on antitumor reactivity, initially causing tumors to grow rapidly, but eventually leading to their complete rejection.

Thus, although this cytokine has complex effects, the induction of lymphocyte IL-10 in the tumor-bearing host is likely to be a critical determinant preventing effective tumor immunity. Furthermore, our data indicate that exposure of DC to IL-10 in vivo may have long-term effects on the capacity of these cells to present Ag. This may limit the effectiveness of autologous DC in clinical trials. Understanding the pathways that regulate tumor-mediated induction of IL-10 will be of central importance in the therapeutic modulation of this immunosuppressive network.

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