Importance of IL-10 for CTLA-4-Mediated Inhibition of Tumor-Eradicating Immunity[1]

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In this study, we show that engagement of CTLA-4 on tumor-infiltrating lymphocytes from low-dose melphalan (L-phenylalanine mustard (L-PAM))-treated MOPC-315 tumor bearers led to IL-10 secretion. In addition, the inhibitory activity of CTLA-4 ligation for IFN-γ secretion following stimulation with anti-CD3 plus anti-CD28 mAb depended on IL-10 production. Consistent with the importance of IL-10 for CTLA-4-mediated inhibition, administration of neutralizing anti-IL-10 mAb to low-dose L-PAM-treated MOPC-315 tumor bearers (administration of blocking anti-CTLA-4 mAb) resulted in enhanced tumor-infiltrating lymphocyte-mediated anti-MOPC-315 cytotoxicity and led to complete tumor eradication in a higher percentage of mice than that observed with low-dose L-PAM alone. The percentage of MOPC-315 tumor-bearing mice cured following administration of neutralizing anti-IL-10 mAb to low-dose L-PAM-treated MOPC-315 tumor bearers was comparable to that observed following administration of blocking anti-CTLA-4 mAb. Moreover, IL-10 neutralization together with CTLA-4 blockade did not provide added therapeutic benefits to low-dose L-PAM-treated MOPC-315 tumor bearers. Taken together, these results indicate that CTLA-4 blockade improves the therapeutic outcome of low-dose L-PAM for MOPC-315 tumor bearers by inhibiting IL-10 secretion as a consequence of blocking CTLA-4 ligation. ***The Journal of Immunology,*** **2004, 172: 1449–1454.**

The demonstration that in vitro blockade of signaling through the CTLA-4 receptor augments T cell proliferation in response to stimulation with alloantigens (1) or anti-CD3 in conjunction with anti-CD28 mAb (1, 2) has generated interest in the use of anti-CTLA-4 mAb in vivo to block the interaction between CTLA-4 and its ligands in order to enhance antitumor immune responses. Indeed, CTLA-4 blockade was used successfully in some tumor models to enhance antitumor immunity and provide therapeutic benefits (3–5). For example, Leach et al. (5) have shown that CTLA-4 blockade inhibited tumor growth as well as caused complete regression of palpable tumors in the 51BLim10 colon carcinoma model and in the Sa1N fibrosarcoma model. Similar observations were subsequently reported by Yang et al. (4) in two other experimental tumor models, the CSA1M fibrosarcoma and the OV-HM ovarian carcinoma.

CTLA-4 blockade alone did not offer any therapeutic benefits in several other tumor models, including the MOPC-315 plasmacytoma (6), the SM1 mammary carcinoma (7), the B16 melanoma (8), and the C2 transgenic adenocarcinoma of mouse prostate (9). Still, the therapeutic benefits of CTLA-4 blockade were realized in MOPC-315 tumor bearers after the administration of a low dose of L-phenylalanine mustard (L-PAM)[4] (6), which led to a shift in the cytokine profile at the tumor site from IL-10 and TGF-β toward TNF-α, IFN-γ, and GM-CSF (10–13).

Several studies into the mechanisms through which CTLA-4 ligation mediates its inhibitory activity for T cell responses have implicated TGF-β in the inhibition (14–17). However, a recent study by Sullivan et al. (18) raised questions regarding the importance of TGF-β for the inhibitory activity of CTLA-4 ligation. Specifically, in this study, Sullivan et al. have shown that CTLA-4 ligation, which did not inhibit the proliferation of T cells from CTLA-4$^{-/-}$ mice, inhibited the proliferation of T cells from TGF-$\beta^{-/-}$ mice or Smad3$^{-/-}$ mice to the same extent as T cells from wild-type mice.

Because L-PAM, which was essential for the realization of the therapeutic benefits of CTLA-4 blockade in the MOPC-315 tumor system, led not only to down-regulation of TGF-β production, but also of IL-10 production (10, 13), the current studies were undertaken to elucidate the importance of IL-10 for the inhibitory activity of CTLA-4 ligation for tumor-eradicating immunity in L-PAM-treated MOPC-315 tumor bearers. In this study, we show that tumor-infiltrating lymphocytes (TILs) from L-PAM-treated MOPC-315 tumor bearers secrete IL-10 following CTLA-4 ligation, and neutralization of IL-10 enhances the CTL activity of the TILs. In addition, we provide data indicating that CTLA-4 blockade improves the therapeutic outcome of L-PAM for MOPC-315 tumor bearers by inhibiting IL-10 secretion as a consequence of blocking CTLA-4 ligation.

Materials and Methods

Tumors

The MOPC-315 plasmacytoma was maintained in vivo, as previously described (10, 13, 19), in BALB/cAnNCrlBR mice 7–10 wk old that were purchased from Charles Rivers Breeding Laboratories (Wilmington, MA). Routinely, mice were inoculated s.c. with 1×10^6 MOPC-315 tumor cells, a dose that is at least 300-fold greater than the minimal lethal tumor dose,

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[4] Abbreviations used in this paper: L-PAM, L-phenylalanine mustard; NIgG, normal IgG; TIL, tumor-infiltrating lymphocyte.

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and leads to the appearance of progressively growing tumors that kill the mice in ~18 days (6). On day 10 after tumor inoculation, when the tumors reached 18–22 mm in diameter, the tumor nodules were excised and single cell suspensions were prepared by mechanical disruption between glass slides.

L-PAM therapy

A fresh stock solution of L-PAM (Sigma-Aldrich, St. Louis, MO) was prepared, as previously described (10, 20). A dose of 0.75–1.25 mg/kg body weight (low dose) was administered i.p. to mice bearing a large (~20-mm) s.c. tumor that resulted from the s.c. inoculation of 1×10^6 MOPC-315 tumor cells 10 days earlier. This dose of drug is curative for ~50% of BALB/c mice bearing a large MOPC-315 tumor, but only in cooperation with host $CD8^+$ T cell-dependent tumor-eradicating immunity that emerges after the chemotherapy (19, 20). In mice in which the low-dose L-PAM is curative, significant regression of the s.c. tumor nodule is evident within 4 days after the L-PAM administration with complete regression of the s.c. tumor nodules within 8–10 days after the chemotherapy (10, 11, 19).

Cross-linking of CTLA-4 on TILs

TILs were obtained, as previously described (10, 20), from the s.c. tumor nodules of mice in which the s.c. tumor nodules regressed from ~20 to ~12 mm as a consequence of low-dose L-PAM administration 5–6 days earlier. The percentage of T cells in these TIL preparations exceeded 90%. Subsequently, 5×10^5 TILs in DMEM supplemented with 5% FBS (Sigma-Aldrich), 5×10^{-5} M 2-ME, 1% nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, and 15 mM HEPES buffer (Invitrogen Life Technologies, Carlsbad, CA) (complete DMEM) were seeded per well of a 96-well flat-bottom tissue culture plate (Falcon; BD Biosciences Discovery Labware, Bedford, MA). Hamster anti-mouse CTLA-4 mAb (UC10-4F10) (1) or purified normal hamster IgG (NIgG; Pierce, Rockford, IL) was then added at a final concentration of 50 μg/ml, and the tissue culture plates were incubated for 10–15 min on ice. This was followed by the addition of 30 μg/ml polyclonal goat anti-hamster IgG Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) and incubation of the plates for 72 h at 37°C. At the end of the incubation period, IL-10 concentration in the supernatants was determined with the aid of an ELISA wherein JES5-2A5 rat anti-mouse IL-10 mAb was used as capture Ab and the SXC-1 rat anti-mouse IL-10 mAb as detection Ab (BD PharMingen, San Diego, CA).

Induction of IFN-γ production

Spleen cells (4×10^5) from wild-type BALB/c mice or IL-$10^{-/-}$ mice (21) on BALB/c background (obtained through the generosity of D. Rennick from DNAX, Palo Alto, CA) were incubated in 0.2 ml complete DMEM in 96-well plates precoated, as previously described (17), with 2 μg/ml anti-CD3 mAb (145-2C11) and 10 μg/ml anti-CD28 mAb (PV-1). Anti-CTLA-4 mAb (20 μg/ml) was added to some of the wells, and 20 μg/ml polyclonal goat anti-hamster IgG was added to all of the wells. To some of the wells we also added 10 μg/ml anti-IL-10 mAb (JES5). The plates were incubated for 48 h at 37°C, at the end of which the supernatants were harvested and evaluated for IFN-γ concentration with the aid of an ELISA wherein the R46A2 rat anti-mouse IFN-γ mAb was used as capture Ab and the XMG1.2 rat anti-mouse IFN-γ mAb as detection Ab (BD PharMingen).

In vivo treatment with mAb.

In experiments assessing the ability of CTLA-4 blockade to offer therapeutic benefits to mice bearing a 20-mm s.c. MOPC-315 tumor and treated with a low dose of L-PAM (0.75–1.25 mg/kg), the mice were given an i.p. injection of 50 μg of anti-CTLA-4 mAb (UC10-4F10) every other day for a total of four injections starting on the day of L-PAM administration. In experiments assessing the ability of IL-10 neutralization to offer therapeutic benefits to L-PAM-treated MOPC-315 tumor bearers with or without anti-CTLA-4 mAb, the mice were given an i.p. injection of 1–2 mg rat anti-mouse IL-10 mAb (JES5 or SXC-1) or rat NIgG (Sigma-Aldrich) on the day of L-PAM administration, followed by a second injection 5 days later.

Antitumor cytotoxicity

TILs isolated from the tumor nodules of mice on day 5 after the administration of low-dose L-PAM in conjunction with anti-IL-10 mAb or rat NIgG were evaluated for their anti-MOPC-315 lytic activity, as previously described (12, 13, 19), by the 3.5-h ^{51}Cr release assay. The percentage of ^{51}Cr released was calculated by the following formula: $((E^{cpm}–S^{cpm})/(M^{cpm}–S^{cpm})) \times 100$, in which E^{cpm} represents the ^{51}Cr released by target cells incubated with effector cells, S^{cpm} represents the spontaneous release, and M^{cpm} represents the maximal release obtained by the addition of 2% Nonidet P-40 detergent.

Statistical analysis

The significance of differences in the fraction of mice surviving after the different treatments was determined by the Gehans-Wilcoxon test. For all other statistical analyses, Student's *t* test was used. A p value of ≤ 0.05 was considered significant in both tests.

Results

Cross-linking of CTLA-4 on TILs from low-dose L-PAM-treated MOPC-315 tumor bearers leads to IL-10 secretion

Experiments were undertaken to determine whether CTLA-4 ligation on TILs from low-dose L-PAM-treated MOPC-315 tumor bearers leads to elevated production of IL-10. For this purpose, TILs were obtained from the tumor nodules of MOPC-315 tumor bearers on day 5 or 6 after the chemotherapy; at a time at which the TILs are actively engaged in tumor eradication (19, 20, 22). The TILs were exposed to hamster anti-mouse CTLA-4 mAb, followed by cross-linking with polyclonal goat anti-hamster Ab, and the level of IL-10 present in the supernatants of the TILs was determined 72 h later. As a negative control, the TILs were exposed to hamster NIgG, followed by goat anti-hamster Ab. As seen in Fig. 1, TILs exposed to hamster anti-mouse CTLA-4 mAb plus polyclonal goat anti-hamster Ab secreted more IL-10 than TILs exposed to hamster NIgG plus goat anti-hamster Ab. Thus, cross-linking of CTLA-4 on TILs from low-dose L-PAM-treated MOPC-315 tumor bearers results in elevated secretion of IL-10.

IL-10 is of primary importance for CTLA-4-mediated inhibition of IFN-γ production

Experiments were conducted to elucidate the importance of IL-10 for CTLA-4-mediated inhibition of IFN-γ production because IFN-γ was previously shown to be important for the curative effectiveness of low-dose L-PAM for MOPC-315 tumor bearers (11). As seen in Fig. 2, activation of spleen cells with anti-CD3 plus anti-CD28 mAb led to the secretion of a substantial level of IFN-γ, which was inhibited as a consequence of CTLA-4 ligation. However, while CTLA-4 ligation resulted in ~70% reduction in the

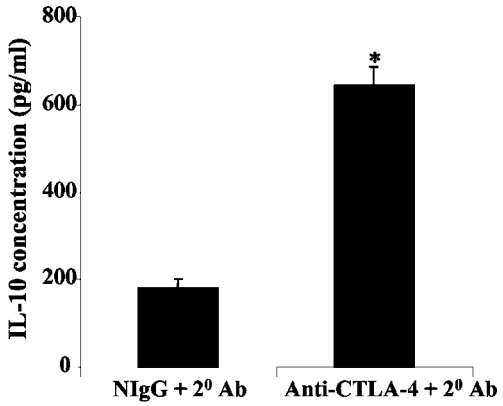

FIGURE 1. Assessment of IL-10 secretion following ligation of CTLA-4 on TILs from L-PAM-treated MOPC-315 tumor bearers. TILs were obtained from the s.c. tumor nodule of mice treated with low-dose L-PAM 6 days earlier. The TILs were cultured for 72 h in the presence of hamster anti-mouse CTLA-4 (anti-CTLA-4) mAb or hamster NIgG and polyclonal goat anti-hamster Ab (2^o Ab). The level of IL-10 present in the culture supernatants was subsequently determined by ELISA. The results of a representative experiment of a total of five experiments are provided. *, Indicates a significantly higher level of IL-10 than that present in culture supernatants of TILs exposed to NIgG plus 2^o Ab.

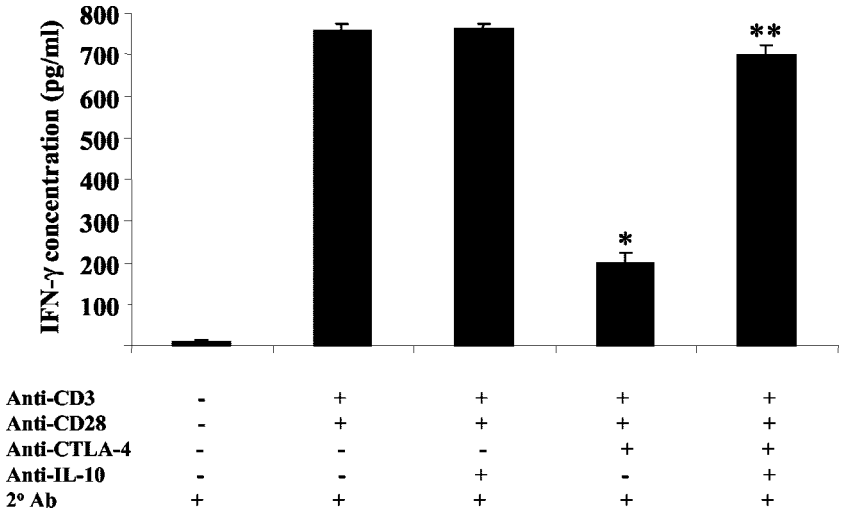

FIGURE 2. Effect of IL-10 neutralization on the inhibitory activity of CTLA-4 ligation for IFN-γ secretion following stimulation of T cells with anti-CD3 plus anti-CD28 mAb. Spleen cells from wild-type mice were exposed to the mAb listed below each bar in the figure and cultured for 48 h. The level of IFN-γ present in the culture supernatants was subsequently determined by ELISA. The results of a representative experiment of a total of five experiments are provided. *, Indicates a significantly lower level of IFN-γ than that present in culture supernatants of cells exposed to anti-CD3 plus anti-CD28 mAb (and 2° Ab). **, Indicates a significantly higher level of IFN-γ than that present in culture supernatants of cells exposed to anti-CD3 plus anti-CD28 in the presence of anti-CTLA-4 mAb (and 2° Ab).

level of IFN-γ produced following activation with anti-CD3 plus anti-CD28 mAb (i.e., from 756 to 198 pg/ml), CTLA-4 ligation in the presence of neutralizing anti-IL-10 mAb resulted in only ~10% reduction in the level of IFN-γ produced by the activated spleen cells (i.e., from 756 to 696 pg/ml). At the same time, addition of neutralizing anti-IL-10 mAb to spleen cells cultured with anti-CD3 plus anti-CD28 mAb (in the absence of anti-CTLA-4 cross-linking) had no effect on the level of IFN-γ secreted (i.e., 756 vs 762 pg/ml). Thus, the inhibitory activity of CTLA-4 ligation for IFN-γ production by the activated spleen cells was almost completely reversed with neutralizing anti-IL-10 mAb.

To confirm our findings with neutralizing anti-IL-10 mAb in experiments using cells from wild-type mice, we conducted experiments with cells from IL-$10^{-/-}$ mice. The experiments with cells from IL-$10^{-/-}$ mice were performed concomitantly with experiments with cells from wild-type mice. A total of four experiments was conducted with spleen cells from IL-$10^{-/-}$ mice, and the level of IFN-γ produced following activation with anti-CD3 plus anti-CD28 mAb ranged between 639 and 1304 pg/ml. The results of a representative experiment are provided in Fig. 3. Although CTLA-4 ligation resulted in a substantial (>50%) reduction in the level of IFN-γ produced by activated spleen cells from wild-type mice, CTLA-4 ligation had only a small (~10%) inhibitory effect on IFN-γ produced by activated spleen cells from IL-$10^{-/-}$ mice (i.e., from 672 to 603 pg/ml). In fact, a similarly small magnitude (<15%) of inhibition of IFN-γ production as a consequence of CTLA-4 ligation was observed in each of the four experiments performed with cells from IL-$10^{-/-}$ mice, regardless of the level of IFN-γ produced by such cells when activated with anti-CD3 plus anti-CD28 mAb in the absence of CTLA-4 ligation. Thus, IL-10 is of primary importance for CTLA-4-mediated inhibition of IFN-γ production as a consequence of activation of T cells with anti-CD3 plus anti-CD28 mAb.

IL-10 neutralization leads to enhancement in the CTL activity of TILs from L-PAM-treated MOPC-315 tumor bearers

Because administration of blocking anti-CTLA-4 mAb to L-PAM-treated MOPC-315 tumor bearers was previously shown to enhance the in vivo acquisition of anti-MOPC-315 cytotoxicity (6), and because IL-10 is shown in this study to be important for CTLA-4-mediated inhibition, we initiated experiments to determine whether administration of neutralizing anti-IL-10 mAb to L-PAM-treated MOPC-315 tumor bearers would also enhance the in vivo acquisition of anti-MOPC-315 cytotoxicity. Specifically, mice bearing a large MOPC-315 tumor were given a low dose of L-PAM in conjunction with either neutralizing anti-IL-10 mAb or NIgG, and on day 5 after the chemotherapy their TILs were obtained and evaluated for anti-MOPC-315 cytotoxicity by the 3.5-h ^{51}Cr release assay. As seen in Fig. 4, TILs from MOPC-315 tumor bearers treated with L-PAM plus neutralizing anti-IL-10 mAb exhibited a higher level of anti-MOPC-315 cytotoxicity than TILs from MOPC-315 tumor bearers treated with L-PAM plus NIgG at any of the E:T ratios used. Thus, IL-10 neutralization, like CTLA-4 blockade (6), enhances the in vivo acquisition of anti-MOPC-315 cytotoxicity by L-PAM-treated MOPC-315 tumor bearers.

IL-10 neutralization provides therapeutic benefits to L-PAM-treated MOPC-315 tumor bearers

Because CTLA-4 blockade was previously shown to provide therapeutic benefits to low-dose L-PAM-treated MOPC-315 tumor bearers (6), we conducted experiments to determine whether IL-10 neutralization would also offer therapeutic benefits to low-dose L-PAM-treated MOPC-315 tumor bearers. For this purpose, we used a dose of L-PAM that is curative for ~50% of the MOPC-315 tumor bearers through a mechanism that requires the participation of $CD8^+$ T cell-mediated tumor-eradicating immunity (6, 11, 19). As seen in Fig. 5, neutralizing anti-IL-10 mAb improved significantly the curative effectiveness of the L-PAM therapy for MOPC-315 tumor bearers. Thus, IL-10 neutralization, like CTLA-4 blockade (6), offers therapeutic benefits to L-PAM-treated MOPC-315 tumor bearers.

IL-10 neutralization combined with anti-CTLA-4 mAb therapy does not provide added therapeutic benefits to low-dose L-PAM-treated MOPC-315 tumor bearers

We next addressed the possibility that CTLA-4 blockade enhances the therapeutic benefits of low-dose L-PAM for MOPC-315 tumor bearers by preventing IL-10 secretion as a consequence of CTLA-4 ligation. For this purpose, we conducted experiments to determine whether IL-10 neutralization, which can offer therapeutic benefits to MOPC-315 tumor bearers treated with low-dose L-PAM alone (Fig. 5), would not be able to offer therapeutic benefits to MOPC-315 tumor bearers treated with low-dose L-PAM plus blocking anti-CTLA-4 mAb. As seen in Fig. 6, neutralizing anti-IL-10 mAb offered similar therapeutic benefits to low-dose L-PAM-treated MOPC-315 tumor bearers as did blocking anti-

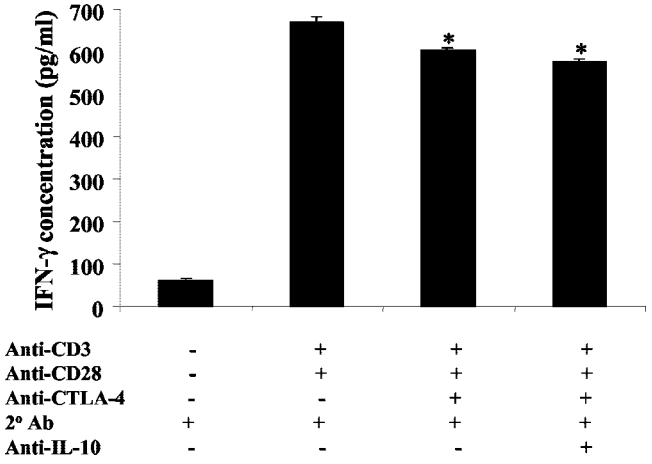

FIGURE 3. Assessment of the ability of CTLA-4 ligation to inhibit IFN-γ secretion by spleen cells from IL-$10^{-/-}$ mice stimulated with anti-CD3 plus anti-CD28 mAb. Spleen cells from IL-$10^{-/-}$ mice were exposed to the mAb listed below each bar in the figure and cultured for 48 h. The level of IFN-γ present in the culture supernatants was subsequently determined by ELISA. The results of a representative experiment of a total of four experiments are provided. In the experiment with spleen cells from wild-type mice performed concomitantly with this experiment, CTLA-4 ligation led to 52% inhibition of IFN-γ production by activated cells (i.e., from 694 to 336 pg/ml). *, Indicates a significantly lower level of IFN-γ than that present in culture supernatants of cells exposed to anti-CD3 plus anti-CD28 mAb (and 2° Ab).

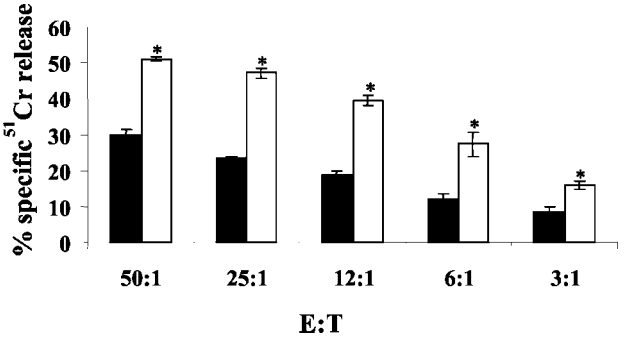

FIGURE 4. Effect of in vivo IL-10 neutralization on the anti-MOPC-315 lytic activity of TILs from mice treated 5 days earlier with low-dose L-PAM. TILs obtained from the tumor nodule of mice treated with low-dose L-PAM plus NIgG (■) or plus neutralizing anti-IL-10 mAb (□) were assessed for their anti-MOPC-315 cytotoxicity by the ^{51}Cr release assay at different E:T. The results of a representative experiment of a total of three experiments are provided. *, Indicates significantly higher level of anti-MOPC-315 cytotoxicity than that exhibited at the same E:T by TILs from mice treated with L-PAM plus NIgG.

CTLA-4 mAb. Moreover, neutralizing anti-IL-10 mAb did not improve the therapeutic benefits offered to low-dose L-PAM-treated MOPC-315 tumor bearers by blocking anti-CTLA-4 mAb, indicating that blocking CTLA-4 mAb improves the therapeutic outcome of low-dose L-PAM for MOPC-315 tumor bearers by preventing IL-10 secretion as a consequence of preventing CTLA-4 ligation.

Discussion

Although it is now well accepted that CTLA-4 ligation exerts an inhibitory effect on T cell responses, the mechanism through which CTLA-4 ligation mediates its inhibitory effect remains unclear (23). Initially, it was proposed that CTLA-4 ligation induces T cell apoptosis, but later on it was found that apoptosis is not a direct result of CTLA-4 ligation, but rather a result of cytokine deprivation as a consequence of CTLA-4 ligation-mediated inhibition of cytokine production (24–26). More recently, TGF-β was implicated in the inhibitory activity of CTLA-4 ligation for $CD4^+$ T cell proliferation and cytokine production. Specifically, Chen et al. (17) have shown that CTLA-4 ligation on $CD4^+$ T cells led to the secretion of elevated levels of TGF-β, and TGF-β in turn was shown to be important for the inhibitory activity of CTLA-4 ligation for $CD4^+$ T cell proliferation. However, although a few studies confirmed the observations of Chen et al. regarding the importance of TGF-β for the inhibitory activity of CTLA-4 ligation for T cell responses (15, 16), other studies did not find TGF-β to play an important role in the inhibitory activity of CTLA-4 blockade (18, 27). Moreover, Sullivan et al. (18) have shown that CTLA-4-mediated inhibition can occur independently of TGF-β, as anti-CD3/CD28/CTLA-4 Ab-coated beads, which did not inhibit the proliferation of T cells from CTLA-$4^{-/-}$ mice, inhibited the proliferation of T cells from TGF-$\beta^{-/-}$ mice or Smad$3^{-/-}$ mice to the same extent as T cells from wild-type mice. However, although the study by Sullivan et al. demonstrated that TGF-β is not important for the inhibitory activity of CTLA-4 ligation, this study did not identify a different regulatory cytokine that may be important for the inhibitory activity of CTLA-4 ligation.

A different regulatory cytokine was identified, however, by Schwarz et al. (27) as responsible for the inhibitory activity of CTLA-4 ligation for T cell responses. Specifically, Schwarz et al. have shown that IL-10 (and not TGF-β) was important for the adoptive transfer of UV-induced tolerance to dinitrofluorobenzene, which was mediated by CTLA-4 ligation. However, this may be unique to the system studied because IL-10, which was shown to be important for the immunosuppressive effects of UV irradiation (28), is induced by UV irradiation (29). In this study, we show that the importance of IL-10 for CTLA-4-mediated inhibition is more general. Specifically, we show that IL-10 is important for CTLA-4-mediated inhibition of IFN-γ production following stimulation of T cells with anti-CD3 plus anti-CD28 mAb. Moreover, we provide data indicating that IL-10 is important for the inhibitory activity of CTLA-4 ligation for the curative effectiveness of low-dose L-PAM for MOPC-315 tumor bearers, under conditions that depend on chemotherapy-induced acquisition of $CD8^+$ T cell-mediated tumor-eradicating immunity.

We would like to point out that in the current studies we have used a very similar protocol to that used by Chen et al. (17) to inhibit T cell responses as a consequence of CTLA-4 ligation, and in fact in both studies CTLA-4 ligation led to a similar magnitude of inhibition of IFN-γ secretion following activation of T cells with anti-CD3 plus anti-CD28 mAb. However, while we reached the conclusion that IL-10 is of primary importance for CTLA-4-mediated inhibition, Chen et al. reached the conclusion that TGF-β is the important regulatory cytokine. Close examination of the data of Chen et al. reveals that although TGF-β played a role in the inhibitory activity of CTLA-4 ligation in their study, the role of TGF-β was rather small. In other words, although in the study by Chen et al. neutralizing anti-TGF-β mAb restored some of the proliferation to $CD4^+$ T cells activated with anti-CD3 plus anti-CD28 mAb in the presence of CTLA-4 ligation, the response observed was still only ~25% of that observed in the absence of CTLA-4 ligation. Thus, most of the inhibitory activity of CTLA-4 ligation in the study of Chen et al. was independent of TGF-β. No information is available in this study with regard to the mediator(s) that was responsible for the majority of the CTLA-4-mediated inhibition. Based on our results, it is conceivable that in the study of

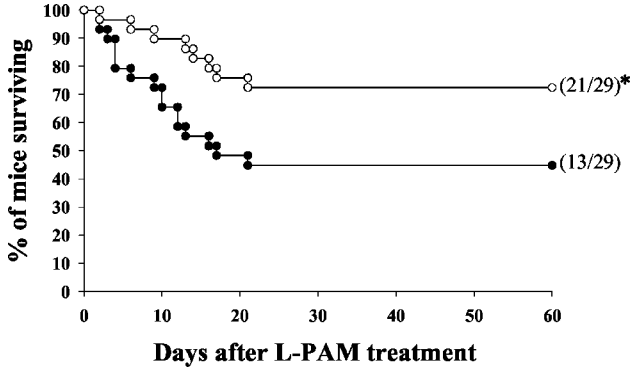

FIGURE 5. Effect of IL-10 neutralization on the curative effectiveness of low-dose L-PAM for MOPC-315 tumor bearers. Mice bearing a large s.c. MOPC-315 tumor were treated with low-dose L-PAM plus NIgG (●) or plus neutralizing anti-IL-10 mAb (○). Numbers in parentheses indicate the number of mice surviving and tumor free out of the total number of mice studied. *, Indicates that the fraction of mice surviving following treatment with L-PAM in conjunction with anti-IL-10 mAb is significantly higher ($p = 0.02$) than that surviving following treatment with L-PAM in conjunction with NIgG.

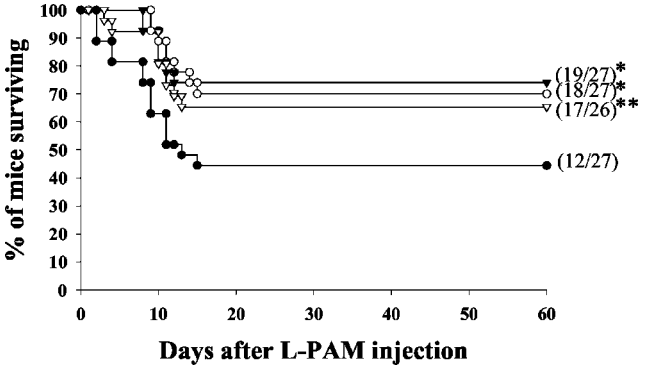

FIGURE 6. Effect of IL-10 neutralization with or without CTLA-4 blockade on the curative effectiveness of low-dose L-PAM for MOPC-315 tumor bearers. Mice bearing a large s.c. MOPC-315 tumor were treated with low-dose L-PAM in conjunction with neutralizing anti-IL-10 mAb (○), blocking anti-CTLA-4 mAb (▼), or both anti-IL-10 and anti-CTLA-4 mAb (▽). As a reference point, another group of mice bearing a large MOPC-315 tumor was treated with low-dose L-PAM in conjunction with NIgG (●). Numbers in parentheses indicate the number of mice surviving and tumor free out of the total number of mice studied. *, Indicates that the fraction of mice surviving following treatment with L-PAM in conjunction with anti-IL-10 or anti-CTLA-4 mAb is significantly higher ($p = 0.01$ or $p = 0.02$, respectively) than that surviving following treatment with L-PAM in conjunction with NIgG. **, Indicates that the fraction of mice surviving following treatment with L-PAM in conjunction with anti-IL-10 and anti-CTLA-4 mAb is higher and approaches significance ($p = 0.0576$) relative to that surviving following treatment with L-PAM in conjunction with NIgG.

Chen et al. IL-10 was responsible for most of the inhibitory activity of CTLA-4 ligation.

It is worth noting that in our studies TILs from L-PAM-treated MOPC-315 tumor did not require activation with anti-CD3 plus anti-CD28 mAb for the elevated production of IL-10 as a consequence of CTLA-4 ligation. This is most likely the result of the strong TCR stimulation that the TILs received in vivo (i.e., just before their in vitro exposure to anti-CTLA-4 plus 2^o Ab), which rendered them active in tumor eradication (19, 20, 22). This explanation is consistent with the observations of Vandenborre et al. (30), showing that human T cells infiltrating skin affected by graft-vs-host disease as well as human T cells infiltrating Hodgkin's disease lesions express CTLA-4 on their surface. In other words, T cells that are actively engaged in carrying out an immune activity (e.g., tumor eradication in our studies or graft-vs-host response in the studies of Vandenborre et al.) may not require exogenous TCR stimulation to observe the effect of CTLA-4 ligation. In addition to the strong antigenic stimulation that TILs from L-PAM-treated MOPC-315 tumor bearers received very recently in vivo, it is possible that the TILs also received some TCR stimulation in vitro (i.e., during the 72-h culture in the presence of anti-CTLA-4 mAb plus 2^o Ab) from residual tumor cells and/or tumor-associated Ags present in the TIL preparations. Regardless of whether antigenic stimulation of the TILs took place only in vivo or also in vitro, CTLA-4 ligation on TILs from low-dose L-PAM-treated MOPC-315 tumor bearers, in the absence of exogenous TCR stimulation, led to IL-10 production. These observations that IL-10 is produced by TILs from L-PAM-treated MOPC-315 tumor bearers as a consequence of CTLA-4 ligation, in the absence of exogenous TCR stimulation, are consistent with our observations that IL-10 is of primary importance for the inhibitory activity of CTLA-4 ligation for IFN-γ production following activation of spleen cells with anti-CD3 plus anti-CD28 mAb (Figs. 2 and 3).

Consistent with the suggested importance of IL-10 for the inhibitory activity of CTLA-4 ligation for tumor-eradicating immunity in low-dose L-PAM-treated MOPC-315 tumor bearers, we show in this work that neutralizing anti-IL-10 mAb enhanced the CTL activity exhibited by TILs from low-dose L-PAM-treated MOPC-315 tumor bearers. Moreover, IL-10 neutralization improved the therapeutic outcome of low-dose L-PAM therapy for mice bearing a large MOPC-315 tumor, under conditions that require the participation in tumor eradication of $CD8^+$ T cell-mediated antitumor immunity that emerges after the chemotherapy (19). Interestingly, the magnitude of improvement in the curative effectiveness of low-dose L-PAM for MOPC-315 tumor bearers as a consequence of administration of neutralizing anti-IL-10 mAb was similar to that observed as a consequence of administration of blocking anti-CTLA-4 mAb, indicating that IL-10 and CTLA-4 ligation exert a similar magnitude of inhibition of tumor-eradicating immunity in L-PAM-treated MOPC-315 tumor bearers. Moreover, neutralizing anti-IL-10 mAb was unable to enhance further the therapeutic benefits offered by blocking anti-CTLA-4 mAb to low-dose L-PAM-treated MOPC-315 tumor bearers, indicating that IL-10 is important for the inhibitory activity of CTLA-4 ligation for the curative effectiveness of low-dose L-PAM for MOPC-315 tumor bearers.

Our observations that IL-10 is important for the inhibitory activity of CTLA-4 ligation for tumor-eradicating immunity in low-dose L-PAM-treated MOPC-315 tumor bearers suggest that CTLA-4 blockade alone may not offer therapeutic benefits in tumor systems in which the tumor cells themselves produce IL-10 because IL-10 production by the tumor cells would continue after IL-10 production by the T cells has ceased (as a consequence of CTLA-4 blockade) and would inhibit antitumor immunity. Consistent with this suggestion, we have previously shown that MOPC-315 tumor cells secrete IL-10 (10), and CTLA-4 blockade alone did not provide any therapeutic benefits to MOPC-315 tumor bearers at any stage of tumor growth (6). To realize the therapeutic benefits of CTLA-4 blockade in the MOPC-315 tumor system, the mice had to be given low-dose L-PAM (6), which abrogated IL-10 production by the MOPC-315 tumor cells (10). In addition, consistent with our suggestion, CTLA-4 blockade alone was shown by Leach et al. (5) to offer therapeutic benefits to mice bearing palpable SalN tumors (5), and we have recently found that SalN tumor cells do not produce IL-10 (data not shown). It is not known

at present, however, whether the lack of IL-10 production is the sole reason for the effectiveness of CTLA-4 blockade alone for the therapy of Sa1N tumor-bearing mice.

Because some human tumors were reported to secrete large amounts of IL-10 (31–33), our results would suggest that CTLA-4 blockade alone would not offer therapeutic benefits to patients with these tumors. This cautionary note is particularly timely as clinical trials with anti-CTLA-4 mAb are currently underway in melanoma patients that did not respond to previous therapy (34, 35), and melanoma cells from a substantial percentage of patients were reported to produce large amounts of IL-10 (31, 32).

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