Interleukin-10 Ablation Promotes Tumor Development, Growth, and Metastasis

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Abstract

Interleukin-10 (IL-10) is a broadly acting immune inhibitory cytokine that is generally thought to support tumor growth. Here we challenge this view with evidence that genetic ablation of IL-10 in the mouse significantly heightens sensitivity to chemical carcinogenesis, growth of transplanted tumors, and formation of metastases. Tumor growth in IL-10-deficient (IL-10⁻/⁻) mice was associated with an increased level of myeloid-derived suppressor cells (MDSC) and CD4⁺Foxp³⁺ regulatory T (Treg) cells in both the tumor microenvironment and the tumor-draining lymph nodes. IL-10⁻/⁻ MDSCs express high levels of MHC and IL-1, and they efficiently induced formation of Treg cells. IL-1 signaling blockade reduced tumor growth mediated by IL-10 deficiency, associated with a partial rescue of tumor infiltration and function of effector T cells and a decrease in tumor angiogenesis and tumor infiltration by Treg cells. Taken together, our findings establish that endogenous IL-10 inhibits inflammatory cytokine production and hampers the development of Treg cells and MDSCs, two key components of the immunosuppressive tumor microenvironment, thereby inhibiting tumor development, growth, and metastasis.

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Introduction

Interleukin-10 (IL-10) was first described as a product of T helper 2 (Th2) cells that inhibited cytokine production by T helper 1 cells (Th1; refs. 1, 2). In addition to Th2 cells, IL-10 is produced by different T-cell subsets (including Th1 cells) (3), B cells, myeloid antigen-presenting cells (APC; refs. 1, 2), keratinocytes (4), and epithelial cells (5). IL-10 has been thought to be largely immunosuppressive, enacting multiple inhibitory effects on APCs. IL-10 restrains antigen presentation via its inhibition of MHC and costimulatory B7 family member molecules (1, 2), stimulates inhibitory B7 family members (6–8), downregulates IL-12 production, and inhibits dendritic cell (DC) differentiation and maturation (9). Therefore, most attention has been devoted to the immunosuppressive roles of IL-10 in a variety of experimental settings, particularly in infectious disease models (9–11). In addition to immunosuppressive effects, IL-10 also exerts some immunostimulatory effects, such as promoting the generation of cytotoxic T lymphocytes, activating B cells (1, 3, 12), and upregulating a small number of genes in TLR-activated macrophages and DCs (13).

Although the underlying mechanisms are poorly understood, the stimulatory effects of IL-10 have also been observed in the context of tumor. Transfection of tumor cells with IL-10 or systemic IL-10 administration significantly suppressed tumor growth and led to tumor rejection (14–17). These data suggest that the biological activities of IL-10 in tumor pathology may be highly context-dependent.

Over the past few years, we and others have achieved important insights into tumor immunopathogenesis in patients with cancer. We and others have shown that the tumor microenvironment is comprised of regulatory T (Treg) cells (18, 19), myeloid-derived suppressor cells (MDSC; refs. 20–28) and dysfunctional APCs (6, 7, 29, 30) that form a suppressive network to defeat tumor-specific immunity and promote tumor growth. Because IL-10 is functionally linked to Treg cells and MDSCs (15–17), we revisited the role of IL-10 in tumor pathology in this study. We have shown that IL-10 inhibits inflammatory cytokine production, hampers the development of MDSCs and Treg cells in the tumor microenvironment, and retards tumor development, growth, and metastasis.

Methods

Tumor models

Six- to 8-week-old C57BL/6 mice (strain #000664) were purchased from the Jackson Laboratory. IL-10⁻/⁻ mice (strain #2251) and IL-1R⁻/⁻ mice (strain #3245) were purchased from...
the Jackson Laboratory (Jackson Laboratory, Maine) and bred in-house. The mice were maintained in specific pathogen-free conditions. This research was approved by the committee on Use and Care of Animals at the University of Michigan. A total of 1 × 10⁶ MC38 mouse colon carcinoma cells were inoculated subcutaneously into the left flank of IL-10⁻/⁻, IL-10⁺/+ or IL-1R⁻/⁻ C57BL/6 mice. Tumor size was measured every 3 days using calipers fitted with a Vernier scale. Tumor volume was calculated based on 3 perpendicular measurements. For other experiments, 2 × 10⁵ MCA310 fibrosarcoma cells were inoculated intravenously into the tail vein of IL-10⁺/+ and IL-10⁻/⁻ mice. At 2 weeks postinoculation, mice were sacrificed and their livers were harvested, perfused with India Ink, and fixed in parafomaldehyde. The numbers of lung foci were quantified with the aid of a magnifier. MC38 was from Dr. Walter Storkus and MCA310 was from Drs. Alfred Chang and Bernard Fox. MC38 was tested in 2009 (31) and MCA310 was tested in 2009 (32) and 2010 (this work) for in vivo tumor formation in mice.

**AOM/DSS treatment**

Mice were given 10 mg/kg azoxymethane (AOM; Sigma) via intraperitoneal injection. Five days later, they were allowed free access to water containing 2% dextran sodium sulfate (DSS; MP Biomedicals/Fisher) for 5 days, followed by 16 days of regular culture. This cycle was repeated twice and mice were sacrificed 2 weeks after the end of the last DSS cycle or at the end of 9 weeks. Colonos were harvested, fixed with paraformaldehyde. Permeabilized tissues were stained with rat anti-mouse CD8 (1:50; BD Pharmingen), and/or rabbit anti-mouse Foxp3 (1:500; Abcam), followed by goat anti-rat and goat anti-rabbit antibodies, then bisected extracellularly with specific antibodies, then fixed and permeabilized with Fix/Perm solution (eBioscience) and finally, stained intracellularly with specific antibodies. Samples were acquired on a special order LSRII flow cytometer (BD Biosciences), and data were analyzed with DIVA software (BD Biosciences; refs. 7, 18).

**Flow cytometry analysis**

Single-cell suspensions were made from spleen, tumor-draining lymph nodes (TDLN), and tumor. Cells were labeled with fluorescence-conjugated antibodies to CD45 (Invitrogen), CD4, CD8, Gr-1, CD11b, CD90, CD115 (all eBioscience), IFNY, MHC I, CD19 (all BD Pharmingen), and/or Foxp3 (eBioscience). For cytokine profiles, the cells were stimulated with 50 ng/mL PMA (Sigma) and 1 μmol/L Ionomycin (Sigma-Aldrich) for 4 hours in the presence of GolgiPlug and GolgiStop (BD Biosciences). Cells were first stained extracellularly with specific antibodies, then fixed and permeabilized with Fix/Perm solution (eBioscience) and finally, stained intracellularly with specific antibodies. Samples were acquired on a special order LSR II flow cytometer (BD Biosciences), and data were analyzed with DIVA software (BD Biosciences; refs. 7, 18).

**Immunofluorescence analysis**

Immunofluorescence analysis was done as previously described (7, 18). Briefly, harvested tissues were frozen in OCT and then fixed with paraformaldehyde. Permeabilized tissues were stained with rat anti-mouse CD8 (1:50; BD Pharmingen), rat anti-mouse CD31 (1:500; BD Pharmingen), and/or rabbit anti-mouse Foxp3 (1:500; Abcam), followed by goat anti-rat and goat anti-rabbit antibodies, then bisected extracellularly with specific antibodies. Samples were acquired on a special order LSRII flow cytometer (BD Biosciences), and data were analyzed with DIVA software (BD Biosciences; refs. 7, 18).

**MDSC suppression and Treg induction**

For the immunosuppressive assay, MDSCs were isolated and sorted with a high-speed sorter (FACSIAria; BD) from tumor in tumor-bearing mice, and responder T cells were isolated from spleen in normal wild-type (IL-10⁺/+). Normal spleen T cells were stimulated for 3 days with anti-CD3 (2.5 μg/mL) and anti-CD28 (1.25 μg/mL) in the presence of different concentrations of MDSCs. Thymidine was added in the last 16 hours. T-cell proliferation was determined by thymidine incorporation. In Treg induction experiments, tumor-associated MDSCs were cultured with normal naive CD4⁺ T cells isolated from lymph nodes in the presence of anti-CD3 (2.5 μg/mL) for 6 days. On day 6, the cells were removed from culture, permeabilized, and stained with antibodies to Foxp3, CD4, CD90, and CD11b. Foxp3 expression in total CD4⁺ T cells was analyzed via fluorescence-activated cell sorting (FACS) gated on CD4⁺ CD90⁻ CD11b⁻ cells.

**Real-time reverse transcriptase PCR**

CD11b⁺ cells were isolated from IL-10⁺/+ or IL-10⁻/⁻ splenic single-cell suspensions with CD11b positive selection MicroBeads (Miltenyi), and CD11c⁺ CD11b⁺ cells were further sorted with FACSIAria, mRNA was isolated with TRIzol (Gibco BRL). Cytokine transcripts were detected by real-time reverse transcriptase PCR as previously described (33). Gene-specific primer pairs and Fast SYBR Green Master Mix (Applied Biosystems) were used in a Multiplex instrument (Eppendorf). Data analysis is based on the Cₗ method, with normalization of raw data to a housekeeping gene (HPRT).

**Statistics**

Most experiments were evaluated using the Mann–Whitney test, with P < 0.05 considered significant. Some cases were evaluated with Student’s t test and quadratic regression model, also with P < 0.05 considered significant. Statistics were carried out in the GraphPad Prism program suite (GraphPad Software, Inc.) and Statistica program suite (StatSoft).

**Results**

**IL-10 deficiency increases tumor incidence, growth, and foci formation**

The immune-inhibitory role of IL-10 has been well defined in numerous experimental settings. However, the in vivo effects of endogenous IL-10 on tumor immune responses and tumorigenesis are not well understood. We compared tumor incidence, growth, and foci formation in IL-10⁻/⁻ mice and IL-10⁺/+ mice. The mice were subjected to administration of DSS and/or AOM, as previously described (34). IL-10⁻/⁻ mice, but not IL-10⁺/+ mice,
mice, treated with DSS developed numerous colon polyps. In the presence of DSS and AOM, colon polyps developed in both IL-10−/− and IL-10+/+ mice. However, there were more polyps in IL-10−/− mice than IL-10+/+ mice (Fig. 1A). We next subcutaneously injected a colon cancer cell line, MC38, into mice and observed tumor growth over a period of 2 weeks. MC38 had accelerated growth in IL-10−/− mice as compared with IL-10+/+ mice as shown by tumor volume measurement (Fig. 1B). This was confirmed by measuring tumor weight at multiple time points (Fig. 1C). Examples of final tumors excised from IL-10+/+ and IL-10−/− mice are shown in Fig. 1D. We further examined the effect of endogenous IL-10 on the development of mouse lung metastatic foci. To this end, MCA310, a methylcholanthrene-induced sarcoma, was intravenously injected into the 2 groups of mice. IL-10−/− mice had more tumor foci in the lungs than IL-10+/+ mice (Fig. 1E). Thus, IL-10 deficiency increases tumor incidence, growth, and lung foci formation.

IL-10 deficiency increases Treg cells in the tumor

We next investigated the well-defined immunosuppressive immune cell subsets in the tumor microenvironment, including Treg cells. The percentages of intratumoral Treg cells during the course of MC38 tumor development were higher in IL-10−/− mice as compared with their wild-type counterparts beginning on day 6 (Fig. 2A and B). There was a significant correlation between tumor weight and percentage of intratumoral Treg cells in both IL-10−/− and IL-10+/+ mice. Furthermore, a quadratic regression analysis revealed that Treg cell levels were higher in IL-10−/− mice than IL-10+/+ mice across different tumor volumes (Fig. 2C). Interestingly, although Treg populations do not differ in the spleens and lymph nodes of unchallenged, tumor-free mice (Supplementary Fig. S1A and B), they were considerably increased in the TDLNs and spleen of tumor-bearing mice on days 12 and 15 (Fig. 2D and E). Larger Treg populations were also observed in TDLNs of IL-10−/− mice bearing MCA310 (not shown). The data
indicated that IL-10 deficiency results in increased Treg cells in tumor-bearing mice.

**IL-10 deficiency increases MDSC in the tumor**

In addition to Treg cells, MDSCs are also an important immunosuppressive component in tumor-bearing hosts. We observed that intratumoral Gr-1\(^+\)CD11b\(^+\) MDSCs were consistently increased in IL-10\(^{-/-}\) mice in comparison with IL-10\(^{+/+}\) mice throughout the duration of tumor growth (Fig. 3A, Supplementary Fig. S2A). Similar to intratumoral Treg cells, a quadratic regression analysis revealed that intratumoral MDSC levels were higher in IL-10\(^{-/-}\) mice than IL-10\(^{+/+}\) mice across different tumor volumes (Fig. 3B). Not surprisingly, MHC I and II expression was increased on TDLN MDSCs (Supplementary Fig. S2B, and not shown) in IL-10\(^{-/-}\) mice. Once again, although MDSC populations do not differ in unchallenged mice (Supplementary Fig. S3, day 0), they were consistently increased in the spleens of IL-10\(^{-/-}\) tumor-bearing mice in comparison with IL-10\(^{+/+}\) counterparts (Supplementary Fig. S3). This trend was not dependent upon tumor weight (Supplementary Fig. S4).

We further examined the roles of MDSCs on T-cell activation and tumor growth. In an *in vitro* immune suppression assay, similar to IL-10\(^{-/-}\) MDSCs, tumor-derived IL-10\(^{-/-}\) MDSCs suppressed T-cell proliferation in a dose-dependent manner (Fig. 3C). To directly test the effects of MDSCs *in vivo*, we completed immune cell adoptive transfusion experiments. We initially irradiated IL-10\(^{-/-}\) mice and infused them with total...
mononuclear or MDSC-depleted spleen T cells from IL-10+/+ or IL-10–/– mice. Subsequently, we subcutaneously injected MC38 tumor cells into these mice and monitored tumor growth. We observed that MDSC depletion resulted in significantly reduced tumor growth in mice receiving either IL-10+/+ or IL-10–/– immune cells. This indicated that IL-10+/+ and IL-10–/– MDSCs mediate immune suppression in vivo. However, although tumor volumes were bigger in irradiated mice
receiving IL-10^−/− than in those receiving IL-10^+/+ immune cells (Fig. 3D), which is in line with our observations of nonirradiated mice (Fig. 1B–D), we showed that after MDSC depletion, tumor volumes were similar in irradiated mice receiving either IL-10^−/− or IL-10^+/+ immune cells (Fig. 3D). The data indicated that MDSCs serve as important suppressive components of the IL-10^−/− immune system in vivo.

We then hypothesized that MDSCs might induce Treg cells in tumor-bearing mice, as previously reported (22). In support of this, we observed that both tumor-derived IL-10^−/− and IL-10^+/+ MDSCs from MC38-bearing mice induced Treg cells. However, Treg cells were more efficiently induced by IL-10^−/− MDSCs, as compared with IL-10^+/+ MDSCs (Fig. 3E). Altogether, our results indicated that IL-10 deficiency increases Treg cells and MDSCs and suggest that increased MDSCs may induce Treg cells in tumor-bearing mice.

**IL-1 contributes to increased tumor growth in IL-10^−/− mice**

After showing the cellular mechanisms which may contribute to increased tumor incidence, growth, and foci formation in IL-10^−/− mice, we further examined the relevant molecular patterns. We showed that myeloid cells from tumor-free mice expressed higher levels of IL-1α and IL-1β in IL-10^−/− than IL-10^+/+ mice (Fig. 4A). We then investigated the role of IL-1 in tumor growth in IL-10^−/− mice. Anakinra, the recombinant human IL-1 receptor antagonist (IL-1Ra), has shown biological efficacy in mice (35). We treated tumor-bearing IL-10^−/− and IL-10^+/+ mice with anakinra or vehicle over a period of 3 weeks and observed that IL-10^−/− mice treated with anakinra had reduced tumor burden (Fig. 4B). Interestingly, tumor growth was comparable in IL-10^+/+ mice treated with anakinra and those treated with PBS vehicle (Fig. 4C). In further support of the relevance of IL-1, we showed that tumor volume
IL-1 blockade decreased tumor growth in IL-1 receptor-deficient (IL-1R−/−) mice as compared with IL-1R+/+ mice (Fig. 4D). It is likely that IL-1 contributed to increased tumor growth in IL-10−/− mice.

**IL-1 blockade enhances effector T-cell tumor infiltration and reduces tumor angiogenesis in IL-10−/− mice**

We next examined tumor-infiltrating T cells in IL-10−/− mice treated with anakinra. Immunofluorescent staining revealed that there were more tumor-infiltrating CD8+ T cells in IL-10−/− mice treated with anakinra than with control (Fig. 5A). The levels of intratumoral IFNγ+ CD8+ T cells were also increased in mice treated with anakinra (Fig. 5B). Although the levels of MDSCs were comparable in the 2 groups (not shown), the numbers of tumor-infiltrating Treg cells were reduced in mice treated with anakinra (Fig. 5C). Furthermore, tumor microvessel intensity and size (Fig. 5D) were reduced by anakinra treatment. Altogether, the data suggested that IL-1 blockade promotes tumor immune surveillance and reduces tumor angiogenesis.

**Discussion**

IL-10 has been thought to be largely immune suppressive. However, to our surprise, chemically induced tumor incidence, transplanted tumor growth, and lung foci formation are increased in IL-10−/− mice. This is associated with a number
of immune phenotypic, inflammatory, and functional signatures.

The prevalence of MDSCs and Treg cells is similar in tumor-free IL-10−/− deficient and wild-type mice. However, there are significantly more MDSCs and Treg cells in IL-10−/− deficient tumor-bearing mice than in wild-type tumor-bearing mice. MDSCs and Treg cells are the most important immunosuppressive components in the tumor microenvironment. Our observations suggest that IL-10 may impact the development of MDSCs and Treg cells in the context of tumor, and in turn, regulate tumor immune responses. Consistent with our observations, earlier studies in mice have shown that IL-10 promoted tumor immunity and led to reduced tumor growth or tumor rejection (15–17). However, the underlying mechanisms by which IL-10 mediates suppression of tumor growth despite its defined immune-inhibitory functions remained elusive. In addition to regulating immunosuppressive components, we do not rule out the possibility that IL-10 directly affects T-cell activation. In line with this, it has been reported that IL-10 may promote CD8 differentiation and expansion (36–38). In fact, humans treated with IL-10 showed a reduction of IL-1 and increased IFNγ (39, 40). Our studies suggest that the biological activities of IL-10 may be highly context dependent and vary in the presence of different cellular targets, phases of immune responses, and disease model systems. A similar example of this phenomenon is MyD88 signaling, which has been implicated in the promotion of cancer in several mouse carcinogenesis models. However, under chronic colitis conditions induced by DSS-induced mucosal damage, MyD88 has a protective role in colon cancer (12).

Given that IL-10 can be immune stimulatory and inhibitory, the balance of these effects may determine whether IL-10 is beneficial or detrimental in a given model system and/or disease stage. The immune-regulatory roles of IL-10 are prominently defined in infectious disease models. In these scenarios, APCs are the immediate and predominant targets of IL-10 (38). Infectious pathogens rapidly activate Toll-like receptors on APCs and induce antigen-specific T-cell priming. IL-10 inhibits the maturation and function of APCs via reduced MHC expression and IL-12 production, and subsequently suppresses T-cell priming and Th1-type responses (1, 2). However, in a tumor-bearing host, where there is no obvious acute phase of immune response (30), tumor-associated antigen (TAA)–specific priming may slowly occur, and IL-10 may preferentially target MDSCs and Treg cells before potent TAA-specific effector T-cell immunity is established (38). This may partially explain the discrepancy of IL-10 biology in the literature.

It has been reported that IL-1 supports the development of MDSCs in tumor-bearing mice (25, 26, 41). In line with this possibility, IL-10−/− myeloid cells express high levels of IL-1, and there are more MDSCs in IL-10−/− tumor-bearing mice. MDSCs promote Treg cell development (22). Consistent with this, we have observed that IL-10−/− MDSCs efficiently suppress T-cell expansion and are stronger inducers of Treg cells than IL-10+/+ MDSCs. It has been suggested that loss of MHC or mutations in MHC expression machinery may result in limited tumor antigen recognition and presentation (42). Interestingly, IL-10−/− MDSCs express high levels of MHC molecules and may efficiently present self-antigens to Treg cells, thus activating and expanding Treg cells as we have observed. This may explain why IL-10−/− MDSCs are superior to IL-10+/+ MDSCs in Treg cell induction. Perhaps most tellingly, depletion of MDSCs in our immune cell-transfer model profoundly reduces tumor volume, showing that the presence of MDSCs has a negative impact on host immunity in vivo. In addition, tumor burden in mice receiving IL-10−/− cells depleted of MDSCs was reduced by a larger volume than in those mice receiving IL-10+/+ cells depleted of MDSCs. This suggests that MDSCs that develop in the absence of IL-10 may mediate more powerful suppressive activities in tumor-bearing mice. However, although IL-1 promotes the development of MDSCs in tumor-bearing mice (25, 26, 41), intratumoral MDSCs were not changed after IL-1Ra administration in IL-10−/− mice, whereas effector T cells were increased and Treg cells were reduced. Further studies will determine why IL-1 blockade did not affect tumor-associated MDSCs.

IL-10 suppresses the expression of inflammatory cytokines in chronic inflammation models (1, 2). IL-10−/− mice develop chronic colitis in conventional conditions (43) and are prone to develop colon tumors under the stimulation of only DSS, or of both DSS and AOM. Although there is no obvious inflammation (including colitis) in IL-10−/− mice when housed in specific pathogen-free facilities, IL-10−/− myeloid cells express high levels of IL-1, which may suggest the presence of microscopic inflammation in these mice. This inflammation may be increased after tumor inoculation or induction. Inflammation is often linked to increased tumor vascularization, and IL-1 has long been known as a proangiogenic cytokine (44) and promoter of tumorigenesis (45). In line with this, IL-1 blockade reduces tumor microvessel density and size in IL-10−/− mice. The data suggests that IL-10 negatively regulates inflammatory cytokine production and directly inhibits inflammation-associated cancer development and growth. However, it is also possible that the reduced density of tumor microvessels may be due to the resulting enhanced T-cell immunity that includes the production of IFNγ, a potent antiangiogenic factor (46). Finally, we have shown that IL-1 blockade reduces tumor growth in IL-10−/− mice. Accordingly, the levels of intratumoral Treg cells are reduced, and the numbers of tumor-infiltrating effector T cells and levels of effector cytokines are enhanced. Altogether, the increased tumor development, growth, and metastasis in IL-10−/− mice result from multiple intertwined mechanisms: increased immunosuppression, enhanced inflammation, and possible reduced effector T-cell function and tumor trafficking in tumor-bearing hosts.

In summary, contrasting the established views, we show that IL-10 is negatively linked to the development of immunosuppressive MDSCs and Treg cells, subverts tumor immune suppression, and in turn, contributes to reduced tumor development, growth, and metastasis. Our data indicate that the biological activities of IL-10 can be highly context dependent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Authors’ Contributions

T. Tanikawa, C.M. Wilke, and I. Kryczek designed the research, conducted experiments, analyzed data, and made the figures; J. Kao, G.Y. Chen and G. Núñez helped with experimental design and research protocol; T. Tanikawa, C.M. Wilke, and W. Zou wrote the article.

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