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Depletion of Regulatory T Cells Facilitates Growth of Established Tumors: A Mechanism Involving the Regulation of Myeloid-Derived Suppressor Cells by Lipoxin A₄

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Regulatory T cells (Tregs) are thought to facilitate tumor development by suppressing protective antitumor immune responses. However, recent clinical and laboratory studies show that Tregs are a favorable element against cancer. In this study, we provide evidence that Tregs have both promoting and inhibiting effects on tumors, depending on the stage of tumor development. By using 0.5 mg cyclophosphamide, we constructed a murine liver cancer model in which Tregs were continuously and selectively depleted. Under such conditions, we found that tumor growth was inhibited at early stages but accelerated later on. Analysis of the tumor microenvironment disclosed that long-term Treg depletion by 0.5 mg cyclophosphamide treatment induced Gr-1⁺CD11b⁺ myeloid-derived suppressor cells (MDSCs). Ablation of MDSCs by anti-Gr-1 Ab blocked Treg depletion-induced promotion of tumor growth. Furthermore, lipoxigenases 5 and 12, two enzymes participating in the biosynthesis of the lipid anti-inflammatory mediator lipoxin A₄, were upregulated or downregulated by Treg depletion or adoptive transfer. Correspondingly, the levels of lipoxin A₄ were increased or decreased. Lipoxin A₄ thus regulated the induction of MDSCs in response to Treg depletion. These findings suggest that Tregs may play different roles at different stages of tumor growth: promoting early and inhibiting late tumor growth. Our study also suggests that the interplay among Tregs, MDSCs, and lipoxin A₄ tunes the regulation of tumor-associated inflammation. The Journal of Immunology, 2010, 185: 7199–7206.

Despite major advances in understanding of the mechanisms leading to tumor immunity, the successful translation of these insights into effective tumor immunotherapy has not been achieved because of numerous immunosuppressive mechanisms (1, 2). Among them, myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) play fundamental roles in blocking antitumor responses (3–9). It has been established that MDSCs and Tregs accumulate in the tumor-bearing host. Nevertheless, the manner in which the accumulation of these cells participates in tumor homeostasis remains elusive. Notably, MDSCs have been reported to induce tumor-specific Treg development, which further enhances tumor immunosuppression (10). To date, whether and how Tregs in turn regulate MDSCs is unclear.

The physiological significance of Tregs lies in their suppression of adverse immune responses, promoting peripheral self-tolerance and avoidance of autoimmunity (11, 12). Under pathological conditions, Tregs seem to prevent the formation of catastrophic inflammation, such as that observed in the tumor microenvironment (13–15). Inflammation is regarded as a pivotal element in tumor initiation, promotion, and progression; and inhibiting inflammation may negatively regulate tumor development. Therefore, if it is presumed that the recruitment of Tregs to tumors inhibits the destructive inflammation, Tregs probably have an intrinsic anti-tumor role by counteracting tumor inflammation. In support of this idea, studies from Erdman et al. (16) have shown that the transfer of Tregs prevents cancer development associated with inflammatory colitis. Thus, the roles of Tregs in tumors are probably complex and not just tumor promoting.

Besides immune regulatory cells, recent studies highlight an endogenous eicosanoid, called lipoxin A₄, participating in the regulation of inflammation (17, 18). Lipoxin A₄ is synthesized locally from arachidonic acid at inflammation sites in a transcellular biosynthesis manner (19). By binding its high-affinity G protein-coupled receptor, lipoxin A₄ strongly inhibits the trafficking of leukocytes to the inflammatory site and stimulates the phagocytosis of apoptotic cells by macrophages (17, 18). Currently, lipoxin A₄ and its analogues are considered as "braking signals" of inflammation. However, the regulation of tumor inflammation by lipoxin A₄ remains unclear. In the current study, we show that the depletion of Tregs in large tumors resulted in the production of lipoxin A₄ and the latter consequently induced the generation of MDSCs, thus promoting tumor growth. These findings suggest that Tregs may play different roles at different stages of tumor growth.
growth, promoting early and inhibiting late tumor growth, and also suggest that the body is capable of developing different strategies against tumor inflammation.

Materials and Methods

Tumor model and treatment protocol

BALB/c mice and BALB/c nude mice, 6 to 8 wk old, were purchased from Center of Medical Experimental Animals of Hubei Province (Wuhan, China) for studies approved by the Animal Care and Use Committee of Tongji Medical College. To establish a tumor model, a BALB/c background H22 hepatocarcinoma tumor cell line (2 × 10^6) was used to s.c. inoculate mice. To deplete Tregs, low doses of cyclophosphamide (CY) were i.p. injected into the mice next day. The injection was continued daily.

Isolation of MDSCs from bone marrow and tumor tissue

Tumor-bearing mice were sacrificed, and their tibia and femur were harvested. After lysis of RBCs, bone marrow cells were fractionated by centrifugation on a Percoll (Amersham Biosciences, Uppsala, Sweden) density gradient as described (15). Cells were collected from the gradient interfaces. Cell bands between 40 and 50% were labeled as fraction 1, between 50 and 60% as fraction 2, and between 60 and 70% as fraction 3. As for tumor MDSCs, large tumors were digested with collagenase and hyaluronidase for 1 h at 37°C and homogenized with seminested slides. After lysis of RBCs, the dissociated cells were underlaid with 5 ml lymphocyte separation solution and centrifuged (2200 rpm for 20 min). Tumor-infiltrating lymphocytes including MDSCs were harvested from the interface for flow cytometric analysis.

Flow cytometric analysis

Tregs were incubated with PE-Cy3–conjugated anti-mouse CD3, PE-conjugated anti-mouse CD4 for surface staining. Cells then were washed, fixed, and permeabilized with Fix-Perm (eBioscience, San Diego, CA) solution for intracellular staining with FITC-conjugated anti-CD25 (PC61.5; eBioscience). Similarly, MDSCs were incubated with allophycocyanin-conjugated anti-mouse CD11b and FITC-conjugated anti-mouse Gr-1 for staining. All Abs were purchased from eBioscience.

In vivo depletion of Tregs and MDSCs

CD4^+CD25^+ Tregs were depleted in vivo by i.p. injection of 100 μg anti-mouse CD25 Ab (PC61.5; eBioscience). MDSCs were depleted in vivo by i.p. injection of 50 μg anti-mouse Gr-1 Ab (RB6-8C5; eBioscience).

RT-PCR and real-time RT-PCR

Cells or tumor tissues were lysed or homogenized with TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA was extracted according to the manufacturer’s instructions. An RT-PCR procedure was used to determine the expression of mRNA (One-step RT-PCR kit; Qiagen, Valencia, CA). The primer sequences were as follows: GAPDH 5′-GGTGAAGTTGTTGCCATCAAC G-3′ (antisense), 5′-CAGTGGAGATGCGGATGATTTGC-3′ (sense); Foxp3 5′-CAGCTGCTCAGTGGGTGAGTCCAGTA-3′ (antisense); 5′-CATTGGCAGGAGTGGTAG-3′ (sense). For real-time RT-PCR assays, the cDNA sequences of all detected genes were retrieved from the National Center for Biotechnology Information database. The primers were designed with the Oligo Primer Analysis 4.0 software (Molecular Biology Insights, Cascade, CO), and the sequences were blasted. Total RNA (100 ng) was used for reverse transcription using Superscript II RNase H reverse transcriptase (Invitrogen) in a volume of 25 μl. Then, 2 μl cDNA was amplified with SYBR Green Universal PCR Mastermix (Bio-Rad, Richmond, CA) in duplicate. The resulting data were analyzed with the comparative CT method for relative gene expression quantification against GAPDH.

Western blot analysis

Tumor tissue homogenates (30 μg total protein) and prestained m.w. markers were separated by SDS-PAGE followed by transfer onto nitrocellulose membranes. The membranes were blocked in TBS with 0.5% of Triton X-100 containing 5% nonfat milk and were probed with anti-inducible NO synthase (iNOS) Ab (Cell Signaling, Danvers, MA) or anti-arginase 1 Ab (Abcam, Cambridge, MA). After incubation with the HRP-conjugated secondary Abs, membranes were extensively washed, and the immunoreactivity was visualized by ECL according to the manufacturer’s protocol (ECL kit; Santa Cruz Biotechnology, Santa Cruz, CA). Other Abs were purchased from Santa Cruz Biotechnology.

ELISA

For the assays of IFN-γ, IL-10, CCL2, and TGF-β, tumor tissues were homogenized in PBS (0.5 ml) containing 100 μm PMSF (Sigma-Aldrich, St. Louis, MO), 1% (v/v) aprotinin (Sigma-Aldrich), 2 μg/ml leupeptin (Sigma-Aldrich), and 1 μg/ml pepstatin (Sigma-Aldrich). After centrifugation, the supernatant was assessed by ELISA kits (R&D Systems, Minneapolis, MN). For PGE2 and lipoxin A4 detection, the mouse serum was assessed by ELISA kits (Lengton Bioscience, Shanghai, China).

Adoptive transfer experiments

Splenocytes were used for the isolation of Tregs with the Regulatory T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions for adoptive transfer through tail vein injection (3 × 10^6 per mouse).

Suppression assay

The suppressive activity of MDSCs was assessed in an anti-CD1 and anti-CD28–mediated proliferation assay of T cells. Briefly, splenocytes (1 × 10^6) from BALB/c mice were cultured in the presence of serial dilutions of irradiated MDSCs in 96-well microplates. [3H]Thymidine was added during the last 8 h of the 72-h culture.

Statistics

Results were expressed as mean values ± SD and interpreted by repeated-measure ANOVA. Differences were considered to be statistically significant when the p value was <0.05.

Results

Tregs are continuously depleted by low-dose CY injection in tumor-bearing mice

Low-dose CY treatment has shown efficacy in selective and transient depletion of Tregs in humans and mice, leading to therapeutic effect against tumors (20–22). However, whether long-term Treg depletion by low-dose CY treatment has a better antitumor effect remains unclear. In this study, we first addressed this question by establishing an animal model with long-term Treg depletion by low-dose CY treatment. One milligram CY has been used as low-dose CY in mice (22, 23). Based on this, we injected 0.25, 0.5, or 1 mg CY daily into H22 hepatocarcinoma cell line-inoculated mice for 14 or 25 d and compared the effect of different doses of CY on Treg depletion. As expected, all three dosages of CY effectively depleted Tregs in spleen and tumor tissue (Fig. 1A, 1B). The efficiency between 0.5 and 1 mg CY was similar and higher than that of 0.25 mg CY (Fig. 1A, 1B). However, we found that 1 mg CY but not 0.25 or 0.5 mg CY daily injection caused weight loss and sickness of mice (data not shown). Further analysis of liver and kidney function indicated that neither 0.25 nor 0.5 mg CY affected these two organs (Fig. 1C). In addition, we also analyzed the spleens and lymph nodes after 25-d 0.25 or 0.5 mg CY treatment and found that the relative total T cell number as well as CD4^+ T cell number was not significantly affected by the injection of 0.25 or 0.5 mg CY (Fig. 1D). Taken together, these data suggested that Tregs can be selectively depleted by 0.25 or 0.5 mg CY daily injection and such injection has no effect on effector T cells in vivo.

Treg depletion inhibits early but promotes late tumor growth

Next, we asked 1) whether long-term Treg depletion suppressed tumor growth and 2) whether 0.5 mg CY dose resulted in a better outcome than 0.25 mg CY, considering that 0.5 mg CY had a stronger Treg depletion efficiency (Fig. 1A). Mice were s.c. inoculated with H22 tumor cells and then daily i.p. injected with 0.25 or 0.5 mg CY from day 1 to day 25. We found that both doses of CY treatment inhibited tumor growth (Fig. 2A). However, although having a better Treg depletion efficiency, 0.5 mg CY treatment did not result in a better suppression of tumor growth. Surprisingly, if we compared the two groups of 0.5 and 0.25 mg
Mice were injected i.p. with 0.25 and 0.5 mg CY once per day for 14 or 25 d. At day 14 or 25, the mice were killed, and the tumors were harvested. Tumor cells were isolated by collagenase digestion. The proportion of CD3+CD4+Foxp3+ Tregs in TILs was determined (Fig. 1C). In addition, tumor-infiltrating lymphocytes (TILs) were isolated. The proportion of CD3+CD4+Foxp3+ Tregs in TILs was determined (Bottom, Bottom). Injection of 0.25 and 0.5 mg CY did not affect the functions of liver and kidney. Mice were injected i.p. with 0.25 and 0.5 mg CY once per day for 30 d. The serum levels of glutamic-pyruvate transaminase (GPT) and creatinine were measured. D, CY 0.25 and 0.5 mg injections did not affect the proportion of total T cell and CD4+ T cells. Mice were injected i.p. with 0.25 and 0.5 mg CY once per day for 25 d. The splenocytes and lymph node cells were stained with anti-CD3 and anti-CD4 Abs. The proportion of CD3+ and CD3+CD4+ T cells were analyzed, respectively. The results were combined from three reproducible experiments.

We found that during the first 15 d, 0.5 mg CY showed a more inhibitory effect; thereafter, 0.25 mg CY showed a better antitumor effect (Fig. 2A). This phenomenon could not be explained by the direct pharmacological activity of CY. CY is a prodruk subject to a series of biological activation steps necessary for its cytotoxic effect. Thus, we took the blood plasma from CY-treated tumor-bearing mice at different time points to culture H22 cells for 48 h. We found that plasma from 0.5 mg CY-treated mice had no effect on H22 cell growth (Fig. 2B) or cell death by trypan blue staining (data not shown). Differently, plasma from high-dose CY (2 mg)-treated mice inhibited H22 cell proliferation (Fig. 2B) and induced tumor cell death as examined by trypan blue staining (data not shown).

To ask why CY treatment had no effect on effector T cells and no pharmacological activity on tumor cells (Fig. 1D and Fig. 2B), this tumor-promoting effect might have been due to Treg depletion by CY treatment. Namely, Tregs might have an inhibitory effect on tumor growth. We then used nude mice to validate this idea, as Tregs are deficient in those mice. We inoculated H22 tumor cells to BALB/c nude mice and treated the mice with 0.5 mg CY from day 1 to day 25 and found that such 0.5 mg CY treatment had no effect on tumor growth in nude mice (Fig. 2D). However, we also used anti-CD25 Ab to deplete Tregs in vivo, as the IL-2R α-chain molecule CD25 is expressed on Tregs and has been used as a target for Treg depletion (24). Consistently, by using anti-CD25 Ab to further deplete Tregs after 16 consecutive days of 0.5 mg CY injection, we observed the accelerated tumor growth (Fig. 2E). Again, these data together indicate that Tregs may have an inhibitory effect on tumor growth, and as a result, Treg depletion promotes tumor development in late tumor.

**Treg depletion induces MDSCs in large tumor**

The Treg depletion-mediated tumor promotion led us to screen in the tumor microenvironment that could be affected by Treg depletion and favor tumor growth. We first tested a panel of inflammation-associated factors, including IFN-γ, IL-6, IL-10, CCL2, TGF-β, iNOS, and arginase 1. Our rationales were 1) Tregs participate in the regulation of inflammation; 2) inflammation is a hallmark of tumorigenesis, which promotes tumor development (13–15); and 3) these factors are involved in tumor inflammation. By using real-time RT-PCR, we found that compared with 16-d depletion, 25-d Treg depletion downregulated the expression of IFN-γ but upregulated IL-6, IL-10, TGF-β, CCL2, iNOS, and arginase 1 (Fig. 3A). The protein levels of these factors, evaluated by ELISA or Western blot (Fig. 3B, 3C), were concordant with the RT-PCR results.

The expression profile of the above inflammation-associated factors fits the promotion and progression phenotype of tumor (25). However, our attention was drawn to the increased expression of CCL2, iNOS, and arginase 1. It is well known that iNOS and arginase 1 can be expressed by MDSCs, which are accumulated in bone marrow and chemotactically to tumors through CCL2/CCL2 pathway (3, 26). Moreover, the induction of Tregs by MDSCs (10) also implies a possibility of Tregs acting on MDSCs. Therefore, we analyzed Gr-1+CD11b+ MDSCs by flow cytometry and found that 25-d Treg depletion by 0.5 mg CY injection increased the proportion of MDSCs in both bone marrow and tumor tissue (Fig. 4A). To clarify whether such increased MDSCs were functional, we conducted an in vitro suppression assay. We found that T cell proliferation was strongly inhibited by the induced MDSCs in response to the stimulation of anti-CD3 and anti-CD28 Abs (Fig. 4B), suggesting that those MDSCs are functional. In line with this, we found tumor-infiltrating T cells from the 25-d Treg depletion group showed a hyporesponsive phenotype by much lower expression of IFN-γ and IL-2 and higher expression of IL-4, IL-10, and TGF-β compared with those from the 16-d Treg depletion group (Fig. 4C). In addition, by comparing the proportion of MDSCs at different time points during Treg depletion, we found that 16-d Treg depletion did not significantly increase MDSCs (Fig. 4D, left). Thus, the induction of MDSCs by long-term Treg depletion seemed to occur at the late stage rather than the early stage of tumor development.

Then, we asked if the induction of MDSCs was due to the absence of Tregs rather than other effects of low-dose CY. For this purpose, we replaced the injection of 0.5 mg CY after day 16 with the injection of anti-CD25 depleting Ab (PC-61) for the continuous Treg depletion. We then analyzed MDSCs on day 25. Consistently,
FIGURE 2. Treg depletion produces a protumor effect in large tumor. A, Long-term Treg depletion by 0.25 or 0.5 mg CY suppressed tumor growth. H22 tumor cells were inoculated s.c. into mice \( (n = 6) \). At day 1, the mice were i.p. injected with 0.25 or 0.5 mg CY once per day for 25 d. The tumor growth was measured. The results were combined from three reproducible experiments. \( p < 0.05 \) compared with 0.25 or 0.5 mg CY group. B, The injection of 0.5 mg CY did not generate direct cytotoxicity to H22 cells. H22 cell-inoculated mice were injected with 0.5 mg CY once per day for 0, 14, or 21 d. Peripheral blood plasma was prepared from mice and used to culture the CFSE-labeled H22 cells. Forty-eight hours later, the proliferation of H22 cells was detected by flow cytometry. C, Tumor growth-promoting effect by late Treg depletion. H22 tumor cells were inoculated s.c. to mice. The next day, mice were divided into four groups and then i.p. injected with either 0.25 or 0.5 mg CY for either 16 or 25 consecutive days. The tumor growth of each group was measured. D, CY 0.5 mg had no effect on tumor growth in nude mice. H22 tumor cells were inoculated s.c. to BALB/c nude mice. At day 1, mice were i.p. injected with 0.5 mg CY once per day for 25 d. The tumor growth was measured. E, Treg depletion by anti-CD25 Ab promotes tumor growth. H22 tumor cells were inoculated s.c. into mice. At day 1, mice were i.p. injected with 0.5 mg CY once per day for 16 d. At day 16, mice were i.p. injected with anti-CD25 Ab once every 3 d four times. The tumor growth was measured.

The injection of PC-61 also increased the proportion of MDSCs compared with that of the isotype control (Fig. 4D, right). To confirm this result, we inoculated H22 tumor cells to nude mice and treated the mice with 0.5 mg CY for 24 d. We analyzed MDSCs in bone marrow and tumor tissue and found that such low-dose CY treatment had no effect on the number of MDSCs under the condition of T cell deficiency in nude mice (Fig. 4E). These data suggested that low-dose CY-induced MDSCs are attributable to depletion of Tregs by CY.

We also clarified whether the tumor-promoting effect of Treg depletion was mediated by MDSCs. Although MDSCs are heterogeneous, Gr-1 is the common marker. Recently, the depletion of MDSCs by anti-mouse Gr-1 Ab (clone RB6-8C5) has been shown by different laboratories, including ours (27–29). Using this approach, we started to i.p. inject anti–Gr-1 Ab into CY-treated, Treg-ablated mice on day 14 then once every 3 d. We found that the depletion of MDSCs by Gr-1 Ab reversed the tumor-promoting effect by Treg depletion (Fig. 4F). Therefore, the long-term Treg depletion results in the induction of MDSCs, which promotes tumor growth.

FIGURE 3. Analysis of inflammation-associated factors after Treg depletion. A–C, The expression of inflammation-associated factors in tumor tissues. H22 tumor cells were inoculated s.c. to mice. At day 1, mice were i.p. injected with 0.5 mg CY once per day for 16 or 25 d. The tumor tissues were used for real-time RT-PCR analysis of Foxp3, IFN-γ, IL-10, CCL2, TGF-β, iNOS, and arginase 1 genes (A) or for ELISA analysis of IFN-γ, IL-10, CCL2, and TGF-β (B) or for Western blot analysis of iNOS and arginase 1 (C).

Treg depletion increases lipoxin A4 production for MDSC induction

Next, we wondered how MDSCs were induced by Treg depletion. Previous studies reported that tumor cells produce stem cell factor (SCF), which is required for MDSC accumulation in the tumor-bearing mice (30). However, we found that Treg depletion had no effect on SCF expression in tumor (Fig. 5A, top). Besides SCF, PGE2, derived from arachidonic acid through cyclooxygenase-catalyzed pathway, may also act as an inducer of MDSCs (31). We thus determined the levels of PGE2 in peripheral blood and found that 25-d Treg depletion did not alter peripheral PGE2 levels (Fig. 5A, bottom), suggesting that PGE2 is not involved in the induction of MDSCs in our model. We then detected another arachidonic acid-derived lipid mediator, lipoxin A4, which is synthesized through lipoxygenase-catalyzed pathway and highlighted as a key regulator of inflammation (17–19). Notably, the levels of lipoxin A4 were gradually increased by Treg depletion (Fig. 5B, top), indicating that lipoxin A4 might be involved in the induction of MDSCs. To test this, we cultured bone marrow cells with the supernatant from a freeze–thaw lysate of tumor cells in the presence or absence of lipoxin A4 or its receptor agonist BML-111. Seventy-two hours later, we collected cells for flow cytometric analysis. The result showed that lipoxin A4 and BML-111
FIGURE 4. Treg depletion induces MDSCs in large tumor. A, MDSCs were induced by Treg depletion. H22 tumor cells were inoculated to mice. At day 1, mice were i.p. injected with 0.5 mg CY once per day for 25 d. The mice were sacrificed, and MDSCs in bone marrow and tumor tissue were isolated and analyzed by flow cytometry as described in Materials and Methods. B, MDSCs induced by Treg depletion possessed the suppressive function. After the above 25-d CY injection, Gr-1-CD11b+ MDSCs were sorted from bone marrow Percoll fraction 2 (Fr.2) cells by FACS. Sorted MDSCs with more than 95% Gr-1-CD11b+ were cocultured with splenocytes at various ratios in the presence of anti-CD3 and anti-CD28 Abs. The T cell proliferation was measured by incorporation of [3H]thymidine. *p < 0.05 compared with the corresponding Fr.2 Gr-1-CD11b+ group. C, Comparison of tumor-infiltrating T cell expression profile between 16-d and 25-d Treg depletion. H22 tumor cells were inoculated s.c. into mice. At day 1, mice were i.p. injected with 0.5 mg CY once per day for 16 or 25 d. TILs from tumor tissue were used for T cell isolation with T cell enrichment column (R&D Systems). T cells were analyzed by real-time RT-PCR for IFN-γ, IL-2, IL-4, IL-10, and TGF-β. D, H22 tumor cells were inoculated s.c. into mice. At day 1, mice were i.p. injected with 0.5 mg CY once per day. Mice were sacrificed at different time points, and bone marrow MDSCs were analyzed by flow cytometry (D, left). Alternatively, mice were first i.p. injected with 0.5 mg CY once per day for 16 d, and then with anti-CD25 Ab once every 3 d four times. Mice were then sacrificed for bone marrow MDSC analysis (D, right). E, H22 tumor cells were inoculated s.c. to nude mice. At day 1, mice were i.p. injected with 0.5 mg CY once per day for 24 d. Mice were sacrificed, and MDSCs in tumor tissue and bone marrow were analyzed. F, Treg depletion-mediated tumor growth is MDSC dependent. H22 tumor cells were inoculated s.c. to mice (n = 12). At day 1, mice were i.p. injected with 0.5 mg CY once per day for 25 d. At day 16, six mice were i.p. injected with anti-Gr-1 or isotype control Ab once every 3 d. The tumor size was measured at indicated time points. The results were combined from two reproducible experiments.

**Discussion**

Several implications may be drawn from the current study. 1) Tregs might act as a double-edged sword in tumor: the protumor effect by inhibiting antitumor immunity and the antitumor effect by preventing tumor inflammation. 2) The relationship of Tregs and MDSCs might be coordinative or restrictive in tumor inflammation, depending on the microenvironment. 3) Endogenous lipoxin A4 might be involved in the regulation of the balance between Tregs and MDSCs.

To study the pathophysiological role of Tregs in vivo, several strategies are adapted to deplete or inactivate Tregs, including genetic mutation of Foxp3 gene, anti-CD25, anti-GITR, or anti-CTLA-4 Ab treatment (32–35). However, the mutation of the foxp3 gene profoundly affects the host immune system from its early development, and CD25, CTLA-4, and GITR are also expressed by other immune cells. Therefore, the selective depletion of Tregs is highly desirable. Recently, low-dose treatment with the chemotherapeutic drug CY has been highlighted for Treg depletion (20–23). In this study, we confirmed and expanded this observation by establishing a repeating Treg depletion model. We show that Tregs are selectively depleted by the continuous injection of 0.5 or 0.25 mg CY. These doses are safe because neither tumor cells nor other cell types were affected, and the functions of liver and kidney...
Treg depletion induces MDSCs through lipoxin A₄ pathway. A, SCF and PGE₂ were not affected by Treg depletion. H₂₂ cells were inoculated to mice. At day 1, the mice were i.p. injected with 0.5 mg CY once per day for 25 d. Tumor tissues were used for SCF detection by ELISA (top). Alternately, the peripheral serum was used to detect PGE₂ levels (bottom). B, Treg depletion increased the levels of lipoxin A₄. H₂₂ tumor cells were inoculated to mice. At day 1, the mice were i.p. injected with 0.5 mg CY once per day. The peripheral serum at different time points was used to detect lipoxin A₄ levels (top). Alternately, 24 d after H₂₂ tumor cell inoculation, mice were adoptively transferred with 5 × 10⁶ splenic Tregs or splenocytes (SP). Twenty-four hours later, the levels of lipoxin A₄ were measured (bottom). C, Lipoxin A₄ induced MDSCs. Bone marrow cells were isolated from normal and tumor-bearing mice. Cells were then cultured with the supernatant from a freeze–thaw lysate of tumor cells in the presence or absence of lipoxin A₄ (Cayman, Ann Arbor, MI) or the receptor agonist BML-111 (BIOMOL, Plymouth Meeting, PA) for 72 h. Cells were harvested for Percoll fractionation, and the Percoll fraction 2 cells were used to analyze Gr-1⁺CD11b⁺ cells by flow cytometry (top). Additionally, we inoculated H₂₂ tumor cells to mice. Ten days later, lipoxin A₄ (100 ng) or BML-111 (20 μg) was i.p. injected to the inoculated or normal mice for 14 consecutive days. Mice were sacrificed, and the tumor tissues were used for SCF detection by ELISA (bottom). D, The expression of lipoxygenases was upregulated by Treg depletion. After the above 25-d CY injection, the expression of LO-5, LO-12, and LO-15 in mice bone marrow cells, spleen, and blood PBMCs was detected by real-time RT-PCR (top). Alternately, 24 d after H₂₂ tumor cell inoculation, mice were adoptively transferred with 5 × 10⁶ splenic Treg cells. Twelve hours later, the expression of LO-5, LO-12, and LO-15 was detected (bottom).

FIGURE 5. Treg depletion promotes large tumor growth.

were not changed in mice tested in this study. Other advantages of this method include the relatively simple technical protocol and low cost. Therefore, we establish an ideal approach for long-term Treg depletion in a murine model.

Tregs accumulate in tumor and peripheral lymphoid organs, but the teleological reason for this accumulation remains unclear. Due to their potent immunosuppression, Tregs are currently considered as a major hurdle for developing antitumor immunotherapy. Many studies show that the ablation of Tregs results in the repression of tumors (36–38), and high frequency of Tregs is associated with a worse prognosis (39–41). However, the inconsistent findings are mounting. In inflammation-associated intestinal tumors, Tregs could prevent cancer development (16); and in gastric and colorectal cancers and certain lymphomas, Treg numbers are an indicator of a favorable prognosis (42–44). In the current study, we found that although they promote early tumor growth, Tregs exert an inhibitory effect on tumors later on. Consistently, Curtin et al. (45) reported that although Treg depletion inhibited or even eliminated small glioblastoma, no improvement in survival was observed when Tregs were depleted 24 d after tumor implantation. Therefore, Tregs might have two opposite effects on tumors at different developmental stages of tumor, and these effects also depend on the tumor microenvironment. From a teleological view, we speculate in this study that the body mobilizes Tregs to control the dangerous inflammation by tumor, however the body has to make a compromise by sacrificing the antitumor immunity, thus leading to both protumor and antitumor effects of Tregs.

One surprising finding in this study is that Treg depletion results in the induction of MDSCs. As early as the 1980s, a correlation between accumulation of immature myeloid cells and immune suppression was recognized in both tumor-bearing mice and cancer patients (46–48). Now, MDSCs are considered to be important in tumor progression (49). An important pathway mediating the immunosuppression function of MDSCs is through educating tumor-specific T cells to become Tregs (10). However, whether and how Tregs act on MDSCs remains unclear. In this study, we find that Tregs in large tumor-bearing mice actually suppress the induction of MDSCs, and Treg depletion strongly induces MDSCs. Although both Tregs and MDSCs primarily mediate immunosuppression, MDSCs belong to immature myeloid cells, which may lose the suppressive function and become inflammatory cells such as macrophages and others. Moreover, tumor-infiltrating MDSCs may employ a nonimmune pathway to promote tumor growth (50). Therefore, it may not be surprising that Treg depletion promotes large tumor growth by inducing MDSCs.

It has been reported that the lipid mediator PGE₂ induces MDSCs in tumor-bearing mice (31). In this study, we found that PGE₂ had little or no role in Treg depletion-induced MDSCs. Instead, lipoxin A₄, another lipid mediator, is involved in the induction of MDSCs after Treg depletion. Lipoxin A₄ is an eicosanoid locally and endogenously synthesized from arachidonic acid by 5-lipoxygenase (5-LO) and 12-LO or 15-LO. Upon Treg depletion, the levels of lipoxin A₄ in peripheral blood were gradually increased in our murine tumor model. Consistently, the expression of 5-LO and 12-LO was also upregulated in peripheral blood, bone marrow, and spleen. More convincingly, the injection of lipoxin A₄ into tumor-bearing or untreated mice strikingly induced the accumulation of MDSCs. Therefore, Treg depletion may lead to MDSC induction by increasing production of lipoxin A₄. Even though a detailed mechanism remains to be elucidated, we hypothesize that the altered inflammatory signals by Treg depletion modulate activities of some transcription factors, leading to the upregulation of lipoxygenases for lipoxin A₄ production.

We should indicate the possible limitations of the approaches used to deplete Tregs in this study. We adapted the low-dose CY approach to deplete Tregs and obtained the experimental results. However, it is still possible that low-dose CY might affect cells other than Tregs, thus influencing the interpretation of the results.
However, we also used anti-CD25 Ab PC-61 for Treg depletion in this study. We stained splenocytes from large tumor-bearing mice (21 d) and found that in CD4+CD25+ T cell subset, 4.8% cells were Foxp3 negative. After the treatment with PC-61, such CD25+ effector T cell population decreased to 0.82% (data not shown).

Thus, although the proportion of CD25+ effector T cells is very low in large tumor-bearing mice, these cells are still affected by PC-61. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor may be introduced into the 3′ untranslated region of mouse Foxp3, resulting in the specific depletion of Tregs by the administration of diphtheria toxin (51). However, this approach is not very suitable for our experiments, because the consecutive injection of diphtheria toxin might cause additional effects on immune cells (52) and on H22 hepatocarcinoma tumor cells (53, 54). Therefore, the really ideal approach for Treg depletion in murine tumor models seems unavailable to date.

In summary, our data show that Tregs play opposite roles in tumor development: promoting early and inhibiting late tumor growth. This might be explained by the observation that Treg depletion increases lipoxin A4 levels, which leads to the induction of MDSCs. On the basis of our findings, we suggest that the interplay among Tregs, MDSCs, and lipoxin A4 tunes the regulation of tumor inflammation and maintenance of a relative homeostasis in the tumor-bearing host.

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Disclosures

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