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*J Immunol* 2010; 185:7199-7206; Prepublished online 10 November 2010; doi: 10.4049/jimmunol.1001876

http://www.jimmunol.org/content/185/12/7199
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, 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 tibias and femurs 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 semisifted 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-Pern (eBioscience, San Diego, CA) solution for intracellular staining with FITC-conjugated anti-mouse Foxp3. Similarly, MDSCs were incubated with allopurinol-cyclohexamide-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'-GGGATACTTGAGCCATCAACG-3' (antisense), 5'-GGGATACTTGAGCCATCAACG-3' (sense); 5'-GATTGTGGGATTGTT-GTTCTG-3' (antisense); Foxp3 5'-CAGCTGTCTACATGGCCCTTAGTCTGT-3' (antisense), 5'-ATTGTGGCAGTGTTGGTAG-3' (antisense). 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 using 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 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 preainted 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 Ab, 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 treatment (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
The proportion of total T cell and CD4+ T cells. Mice were injected i.p. anti-CD3, anti-CD4, and anti-Foxp3 Abs, and the proportion of CD3+evident in the 0.5 mg CY group (Fig. 2C). CY, 0.5 mg CY treatment accelerated tumor growth after 16 d (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 for those molecules and cells 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 tumorgenesis, 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 chemoattracted to tumors through CCL2/CCL22 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,
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 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 promoted tumor growth. H22 tumor cells were inoculated s.c. to normal 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.

**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).

**FIGURE 4.** 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
both increased the proportion of Gr-1CD11b+ MDSCs (Fig. 5C, top). To validate this in vitro data in vivo, we injected lipoxin A4 or BML-111 to naive or tumor-bearing mice daily for 7 d. Consistently, the administration of lipoxin A4 or BML-111 could induce MDSCs in either naive or tumor-bearing mice (Fig. 5C, bottom), suggesting that Treg depletion may induce MDSCs by increasing lipoxin A4 production.

Finally, we clarified the regulation of lipoxin A4 by Treg depletion. After 25-d Treg depletion in tumor-bearing mice, we determined the expression of lipoxygenases 5 and 12, two critical enzymes for lipoxin biosynthesis (19). In line with the increased levels of lipoxin A4, we found that these two enzymes were upregulated in bone marrow, spleen, and blood (Fig. 5D, top), suggesting the potential regulation of lipoxin production by Treg depletion. We then used a comparable approach to confirm this. We isolated Tregs and adoptively transferred these cells to large tumor-bearing mice. Twenty-four hours later, we measured the levels of lipoxin A4. The result showed that the levels of lipoxin A4 were decreased by the adoptive transfer of Treg cells (Fig. 5B, bottom), concomitant with the downregulated expression of lipoxygenases 5 and 12 (Fig. 5D, bottom). Taken together, these data suggested that Treg depletion induces MDSCs by regulating lipoxin A4 production.

**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...
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 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 diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria toxin receptor to generate chimeric molecules. This might also influence our interpretation of the results more or less. In addition, it is reported that diphtheria tox