

3-Methylcholanthrene-induced transforming growth factor- β -producing carcinomas, but not sarcomas, are refractory to regulatory T cell-depletion therapy

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(Received October 29, 2009/Revised December 6, 2009/Accepted December 7, 2009/Online publication March 4, 2010)

Regulatory T cells (Tregs) are major immunosuppressors in tumor-bearing hosts. Although Treg-depletion therapy has been shown to induce a complete cure in tumor-bearing mice, this treatment is not always successful. Using 3-methylcholanthrene-induced primary mouse tumors, we examined the distinct regulation of Treg-mediated immunosuppression between carcinomas and sarcomas. We showed that the number of Tregs was greatly increased in squamous cell carcinoma (SCC)-bearing mice compared with sarcoma-bearing mice. This appeared to be because SCC produced higher levels of active transforming growth factor (TGF)- β , which is essential for inducing Tregs, compared with sarcoma. Moreover, SCC, but not sarcomas, were refractory to Treg-depletion therapy by treatment with anti-CD25 mAb. The refractoriness of SCC against Treg-depletion therapy was due to the rapid recovery of Tregs in SCC-bearing mice compared with sarcoma-bearing mice. However, combination treatment of anti-TGF- β mAb with anti-CD25 mAb caused a significant reduction in Treg recovery and induced a complete cure in SCC-bearing mice. Thus, we showed the refractoriness of mouse carcinoma against Treg-depletion therapy using anti-CD25 mAb treatment. We also proposed a novel Treg-blocking combination therapy using anti-CD25 mAb and anti-TGF- β mAb to induce a complete cure of tumor-bearing hosts. (*Cancer Sci* 2010; 101: 855–861)

The accumulative evidence indicates that it is important to consider two immunological aspects for developing an efficient tumor immunotherapy. First, immunological help, such as an efficient adjuvant provoking type 1 immunity, should be introduced into tumor bearers for inducing and maintaining tumor-specific CTLs.⁽¹⁾ Second, immunosuppressions in tumor bearers should be removed to induce full activation of tumor-specific CTLs.^(1–5) Several immunosuppressive mechanisms in tumor bearers have been evaluated in animal experiments.^(2,3,5–10) However, the mechanisms evaluated in mice were not always applicable to clinical study.^(11,12) The difficulties arose because: (i) many investigators used long-term cultured mouse tumor cell lines, possessing distinct properties from primary tumors; and (ii) most primary mouse tumors used for experiments are 3-methylcholanthrene (MCA)-induced sarcoma, even though more than 98% of human cancer consists of carcinoma, because it has been difficult to establish MCA-induced carcinomas. Therefore, it is necessary to use primary mouse carcinoma to define their immunosuppressive tumor escape mechanisms, which might provide some light for evaluating the mechanisms underlying human carcinoma-induced immunosuppression.

In previous published work,⁽¹³⁾ we established a novel carcinogenesis model to induce both squamous cell carcinoma (SCC)

and fibrosarcoma by a single intradermal (i.d.) injection of MCA. Using this method, we could easily and quickly obtain two different types of primary tumors, SCC and sarcoma. Thus, now, it becomes possible to investigate the distinct immunosuppressive mechanisms between primary-induced carcinoma and sarcoma.

Regulatory T cell (Treg)-mediated immunosuppression is one of the major obstacles to curing tumors by immunotherapy.^(2,5,6,14–28) Many investigators have tried to attenuate Treg-mediated immunosuppression by targeting therapy using Treg-associated molecules such as CD25, CTLA-4, GITR, transforming growth factor (TGF)- β , FR4, OX40, and TLR8.^(14–26) Although Treg-depletion therapy by injecting anti-CD25 mAb is a major strategy to remove Treg-mediated immunosuppression, this method is not always successful in inducing tumor rejection.^(15,16,27,28) However, it remains poorly understood why some tumors were refractory to Treg-depletion therapy and escaped from host antitumor defense mechanisms.

Here, we show that MCA-induced primary carcinomas are refractory to Treg-depletion therapy, whereas MCA-induced primary sarcomas are sensitive to it. This is because Tregs are rapidly recovered in carcinoma-bearing mice by active TGF- β , which is produced at high levels by carcinoma compared with sarcoma. Based on these findings, we show that combination treatment of SCC-bearing mice with anti-CD25 mAb and anti-TGF- β mAb caused a complete cure of tumor-bearing mice by blocking the rapid recovery of Tregs. Thus, our paper initially indicates that there is a distinct regulation of Treg-mediated immunosuppressive tumor escape between carcinoma and sarcoma.

Materials and Methods

Mice and tumor cell line. BALB/c mice were obtained from Charles River Laboratories Japan (Yokohama, Japan). All mice were female and were used at 6–8 weeks of age. SCCs and fibrosarcomas were obtained as described in our previous paper.⁽¹³⁾ In brief, we injected 500 μ g MCA i.d. When the tumor reached >100 mm³, we minced the tumor and seeded to a 10 cm dish. The cells were subcultured when they became confluent. We characterized cytokeratin-positive CMC-1 and CMC-9 as SCC, and vimentin-positive CMS-G2 and CMS-G4 as sarcoma by immunohistochemistry.⁽¹³⁾ We cultured SCCs and sarcomas in RPMI-1640 medium (Sigma-Aldrich, Tokyo, Japan) containing 10% FCS (Nichirei Bioscience, Tokyo, Japan) and used within 10 passages. Mink lung epithelial cell line Mv1Lu was donated by the Cell Resource Center for Biomedical Research,

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Institute of Development, Aging and Cancer (Tohoku University, Japan) and was cultured in Minimum Essential Medium Eagle medium (M4655; Sigma-Aldrich) containing 10% FCS.

Reagents. Anti-Foxp3 polyclonal Ab for immunohistochemical staining was kindly donated by Dr. K. Matsushima (Tokyo University, Tokyo, Japan).⁽²⁹⁾ Anti-Foxp3 mAb (150D/E4) used with anti-Ki67 mAb, and anti-Foxp3 mAb (FJK-16S) used along with anti-CD4 mAb were purchased from eBioscience (San Diego, CA, USA). FITC-CD4 mAb (GK1.5) was purchased from BD Biosciences (Fukushima, Japan). Anti-Ki-67 mAb (TEC-3) was purchased from Dako Japan (Tokyo, Japan) and its control IgG mAb was purchased from BD Biosciences. Anti-TGF- β mAb (1D11), its control IgG mAb (13C4), and anti-CD25 mAb (PC61) were purified by Kitayama Labes (Nagano, Japan), and the hybridomas were purchased from American Type Culture Collection (Manassas, VA, USA). Control IgG mAb of PC61 was purchased from BD Biosciences. Recombinant human (rh)TGF- β 1 was purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant human interleukin (IL)-2 was kindly donated by T. Yamada (Shionogi & Co., Osaka, Japan).

Immunohistochemical staining. Snap-frozen lymph nodes embedded in Tissue-Tek OTC Compound (Sakura Finetek, Tokyo, Japan) were cut at 5–7 μ m, fixed for 10 min in cold acetone and used for immunohistochemical analysis. We used an already established double-immunofluorescence staining protocol for Foxp3 and CD4.⁽²⁹⁾ Briefly, the primary antibodies were diluted in 1% BSA (polyclonal rabbit Ab against murine Foxp3 at a concentration of 5 mg/mL and anti-CD4 mAb [diluted 1:500]). The secondary Ab was applied at a 1:500 dilution (anti-rat IgG-Alexa 546, anti-rabbit IgG-Alexa 488; Alexa, Leiden, the Netherlands). The samples were then analyzed with an Olympus FV500 confocal laser scanning microscope system (Olympus Optical, Tokyo, Japan).

³[H]-thymidine incorporation assay. Cell proliferation was evaluated as described previously.⁽¹⁾ Briefly, ³[H]-thymidine was added to culture medium for 4 h and the radioactivity incorporated in cells was measured as a representation of proliferation of the T cells.

Mv1Lu assay. We detected the levels of active TGF- β in tumor supernatant (sup) by slightly modifying a method described in a previous paper.⁽³⁰⁾ In short, Mv1Lu (5×10^3) were scattered in a 96-well flat plate (Nunc, Roskilde, Denmark) in 100 μ L Minimum Essential Medium Eagle. We removed 50 μ L supernatant 12 h later and added 50 μ L diluted tumor supernatants. Then, 24 h later, Mv1Lu growth inhibition was evaluated in a ³[H]-thymidine incorporation assay. We defined the inhibition rate (%) as an inhibition index of active TGF- β . The inhibition rate was calculated as: Inhibition rate (%) = $100 \times [\text{count per minute (cpm) without sup} - \text{cpm with sup}] / [\text{cpm without sup}]$, where cpm is count per minute. In preparing sup, tumor cells (2×10^5) were scattered in a 12-well plate (Corning, Tokyo, Japan) and changed the supernatant 12 h later to fresh medium of 1 mL. The supernatants were collected 24 h later and used after exerting SpinX (Corning).

Antibody staining and flow cytometry. For analyzing the cell growth of Treg cells, we targeted Ki-67, which was expressed in dividing cells. Lymphocytes from the draining lymph node (DLN), distal LN, and spleen cells were treated with fixation/permeabilization buffer attached with a Foxp3 staining set (eBioscience). Two hours later, they were washed with permeabilizing solution buffer and stained with anti-Ki67 mAb (50 μ g/mL) for 20 min. Cells were washed with permeabilizing solution buffer and stained with anti-rat IgG-Alexa 488 for 20 min. Cells were washed again with permeabilizing solution buffer and stained with phycoerythrin-conjugated anti-Foxp3 mAb (150D/E4). FITC-CD4/phycoerythrin-Foxp3 staining was carried out according to the manufacturer's instructions. Data were acquired on a Becton Dickinson FACSCalibur (Becton

Dickinson, Franklin Lakes, NJ, USA). Data were analyzed using CellQuest software (Becton Dickinson).

Treg-depletion therapy model. Anti-CD25 mAb (50 μ g/head; PC61) or control rat IgG was injected i.v. 24 h before tumor inoculation. Anti-TGF- β mAb (500 μ g/head; 1D11) or control mouse IgG (13C4) was given i.p. simultaneously with tumor inoculation and another five times at 2-day intervals. The tumor cells (2×10^6 or 2×10^5) were inoculated i.d. in the left flank. The axillary LNs were collected and analyzed as DLNs. Tumor volume was calculated by the following formula: tumor volume = $0.4 \times \text{length (mm)} \times [\text{width (mm)}]^2$.

Statistical analyses. Mean values and SD were calculated for data from representative experiments and are shown in the figures. Significant differences in the results were determined by Student's *t*-test. *P* < 0.05 was considered significant.

Results

Numbers of Foxp3⁺ CD4⁺ T cells increased in DLN, but neither distal LN nor spleen in MCA-induced carcinoma-bearing mice.

We established a novel carcinogenesis model to induce both sarcoma and SCC by a single i.d. injection of MCA into BALB/c mice, as described in 'Materials and Methods'.⁽¹³⁾ Using this simple and valuable method, we investigated the functional regulation of Tregs in SCC-bearing hosts. We first analyzed the changes in absolute numbers of Foxp3⁺ CD4⁺ Tregs during tumor growth. The absolute numbers of Tregs in DLN, but neither spleen nor distal LN, gradually increased within 16 days after inoculation of the SCC cell line, CMC-1 (Fig. 1A). We also confirmed a similar increase of Tregs in DLN, but not distal LN or spleen, by immunohistochemical analysis (Fig. 1B). In tumor tissue, Tregs were scattered in low density over the tumor area (Fig. S1).

We further addressed whether Tregs existing in DLN, distal LN, and spleen had differing abilities to suppress T cell responses. Purified CD4⁺ CD25⁺ T cells from DLN, distal LN, and spleen were co-cultured with naïve CD4⁺ T cells in the presence of anti-CD3 mAb, and cell growth was measured. As shown in Figure S2, functional differences were not observed between the Tregs prepared from each organ.

Squamous cell carcinoma but not sarcoma induced a great increase of absolute numbers of Tregs in tumor DLN. We established several MCA-induced primary sarcoma and carcinoma cell lines,⁽¹³⁾ and used them for experiments within 10 passages. To investigate the different regulation of Treg-mediated immunosuppression between MCA-induced sarcoma and SCC, we examined the increase of Tregs in DLN of mice bearing carcinoma or sarcoma. Two weeks after the inoculation of 2×10^6 sarcoma cells (CMS-G2 and CMS-G4) or SCC (CMC-1 and CMC-9), the numbers of Tregs in DLN were counted immunohistochemically. Interestingly, mice bearing SCC, but not sarcoma, showed a great increase in the numbers of Tregs (Fig. 2). There was no significant difference in the tumor volume between SCC and sarcoma (data not shown). These results indicated that MCA-induced primary SCC, but not sarcoma, promoted the expansion of Tregs in DLN.

Superior induction of Tregs in SCC-bearing mice by active TGF- β , highly produced by SCC but not sarcoma. In previous work,^(20,21) it was shown that TGF- β was essential for the induction of Foxp3⁺ Tregs *in vitro*, and we also confirmed it (data not shown). In addition to such Treg regulation activity *in vitro*, we showed that TGF- β had Treg regulation activity *in vivo* (Fig. 3A,B). When BALB/c mice inoculated with CMC-1 cells (2×10^6) were treated with anti-TGF- β mAb (1D11) by i.p. injection four times at 2-day intervals for 10 days, the numbers of Tregs in DLN of the tumor-bearing mice significantly decreased compared with those of control IgG-treated mice (Fig. 3A). Similar results were also obtained in immunohisto-

Fig. 1. Regulatory T cells (Tregs) were increased in draining lymph node (DLN), but not distal LN or spleen, in squamous cell carcinoma (SCC)-bearing host mice. (A) CMC-1 (2×10^6 ; representative SCC) was inoculated intradermally. The numbers of CD4⁺ Foxp3⁺ T cells in tumor-side axillary DLN (filled circle), contralateral axillary LN (distal LN; open circle), and spleen (filled triangle) was analyzed 0, 4, 8, 12, and 16 days after CMC-1 inoculation. The data shown are mean \pm SD of three mice in each experimental group. * $P < 0.05$ DLN versus distal LN. Similar results were obtained in two separate experiments. (B) Tregs were detected in LN and spleen of normal mice, and DLN, distal LN, and spleen of tumor-bearing mice by immunohistochemical analysis. Representative data are shown. Green, Foxp3; red, CD4. Magnification, $\times 200$.

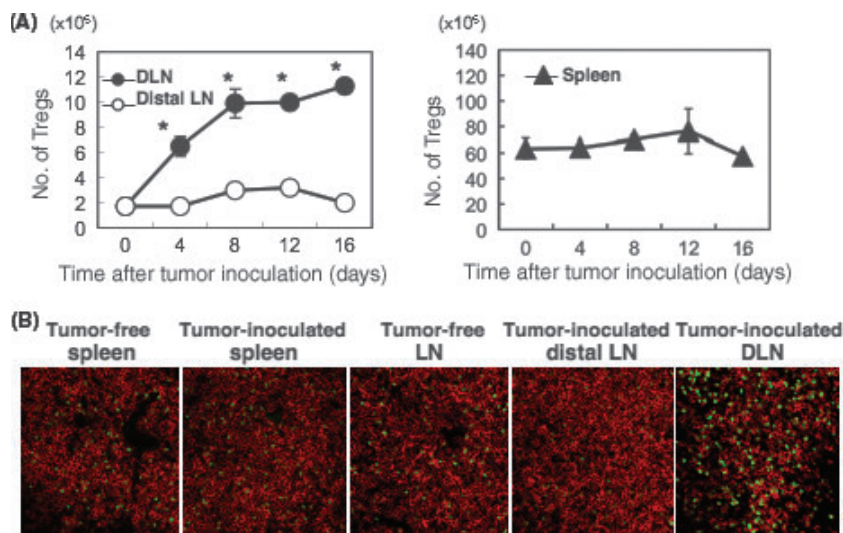
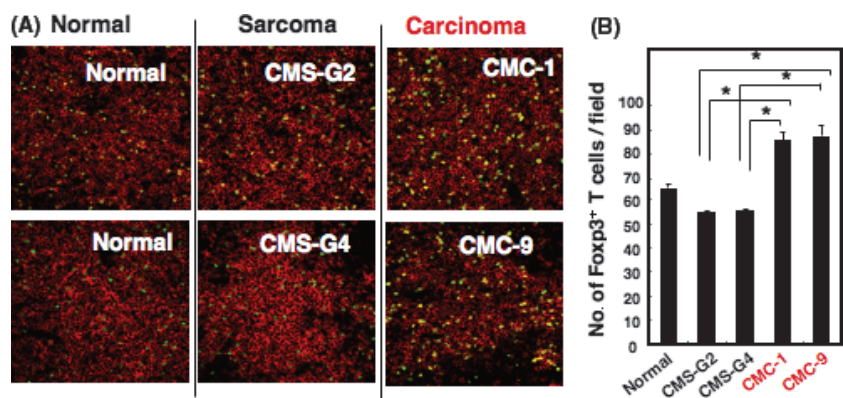


Fig. 2. The number of regulatory T cells (Tregs) in draining lymph node (DLN) increases in 3-methylcholanthrene (MCA)-induced squamous cell carcinoma (SCC)-bearing mice, but not in sarcoma-bearing mice. (A) BALB/c mice were inoculated intradermally with primary sarcoma lines (CMS-G2 and CMS-G4; 2×10^6) and primary SCC lines (CMC-1 and CMC-9; 2×10^6). DLN of the tumor-bearing mice were immunostained with anti-CD4 mAb and anti-Foxp3 mAb. Distributions of CD4⁺ Foxp3⁺ Tregs in the tumor DLN were evaluated by immunohistochemical observation. Green, Foxp3; red, CD4. Magnification, $\times 200$. (B) The numbers of Foxp3⁺ Tregs of the sections were counted. The data shown are mean \pm SD of five fields of each DLN. * $P < 0.02$ sarcoma versus SCC.



chemical analysis (Fig. 3B). These results indicated that TGF- β plays a pivotal role for the induction of Tregs in SCC-bearing mice. Therefore, it was speculated that Treg accumulation in SCC-bearing mice might be due to their ability to produce higher levels of active TGF- β , compared with sarcoma-bearing mice. To demonstrate our working hypothesis, the producibility of active TGF- β from SCC and sarcoma was determined by Mv1Lu assay. Several kinds of primary SCC or sarcoma lines were cultured and TGF- β activity in their culture supernatants was measured, as described in 'Materials and Methods'. Interestingly, most SCC lines showed a higher ability to produce active TGF- β than sarcoma lines (Fig. 3C). These results indicated that a higher ability to produce active TGF- β in MCA-induced carcinoma might explain why larger numbers of Tregs were increased in DLN of carcinoma-bearing hosts compared with sarcoma-bearers.

Carcinoma induced by MCA more resistant to anti-CD25 mAb treatment than sarcoma following a rapid recovery of Tregs. In order to assess the immunosuppressive effects of Tregs on anti-tumor immunity *in vivo*, we depleted Tregs by treatment with anti-CD25 mAb before inoculation of several SCC lines or sarcoma lines at 2×10^6 cells/mouse. Surprisingly, anti-CD25 mAb showed no significant effect on the growth of SCCs, but it completely inhibited the growth of all sarcoma lines through the elimination of Treg-mediated immunosuppression (Fig. 4A,B). For analyzing the regulation of Tregs between both types of tumor-bearing mice, we selected representative SCC (CMC-1) and sarcoma (CMS-G4), which showed contrasting TGF- β production (Fig. 3C) and almost the same growth rates *in vitro* and

in vivo (data not shown). We counted the number of Tregs in CMS-G4- and CMC-1-bearing mice after treatment with anti-CD25 mAb. As shown in Figure 5, tumor-free mice treated with anti-CD25 mAb had a great reduction in naturally occurring Tregs ($0.9 \pm 0.2 \times 10^5$). The decreased numbers of Tregs were slightly recovered ($2.6 \pm 0.6 \times 10^5$) 7 days after the inoculation of 2×10^6 CMS-G4 sarcoma cells. However, in contrast with the case of sarcoma, mice inoculated with 2×10^6 or 2×10^5 CMC-1 cells, which could not be rejected, showed a rapid and significant recovery of Tregs in tumor DLN ($8.9 \pm 0.4 \times 10^5$ and $7.2 \pm 1.0 \times 10^5$). Thus, SCC induced a rapid recovery of Tregs in DLN of their bearing mice compared with sarcoma-bearing mice after Treg-depletion therapy.

Mice bearing SCC resistant to Treg-depletion therapy cured from tumor by combined treatment with anti-TGF- β mAb. The results of Figure 5 suggested that SCC-derived TGF- β might accelerate the recovery of Tregs after depletion, which might induce refractoriness to anti-CD25 mAb treatment. To examine the critical role of TGF- β in the rapid recovery of Tregs, we gave anti-TGF- β mAb (1D11) to CMC-1-bearing mice after injection of anti-CD25 mAb (PC61). As shown in Table 1, the numbers of Tregs were recovered ($1.9 \pm 0.3 \times 10^5$) in CMC-1 bearing mice after treatment with anti-CD25 mAb (PC61) alone. However, the recovery were significantly inhibited ($1.1 \pm 0.1 \times 10^5$) when mice were treated with anti-CD25 mAb (PC61) and anti-TGF- β mAb (1D11) 5 days after tumor inoculation. To examine the antitumor effect of the combination therapy, we next evaluated tumor growth in mice treated with both mAbs. As shown in Figure 6, CMC-1-bearing mice treated with anti-

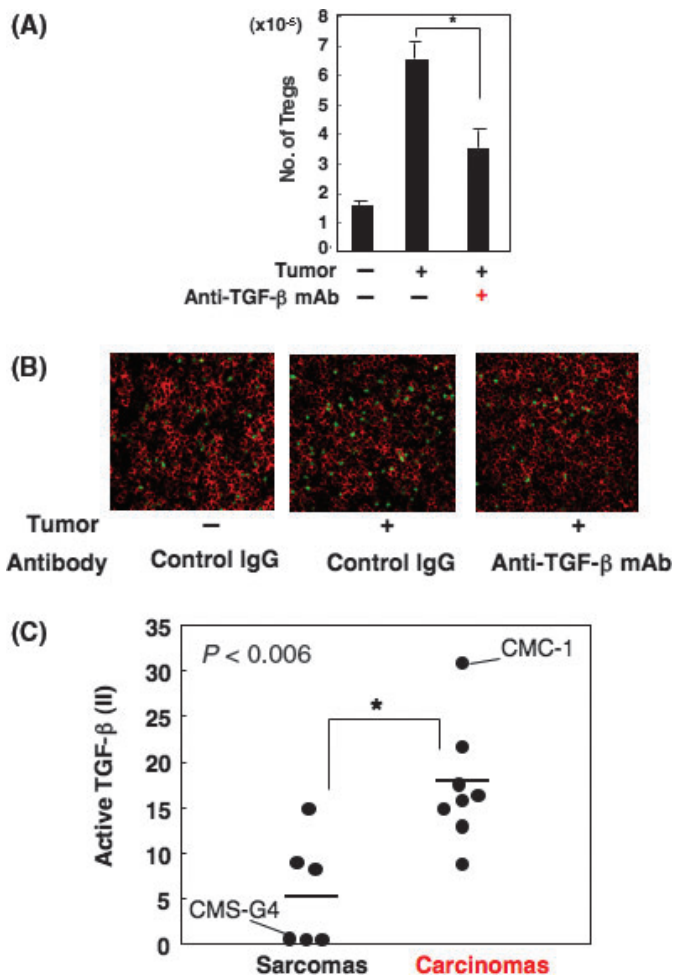


Fig. 3. Squamous cell carcinomas (SCCs) predominantly induce regulatory T cells (Tregs) by their ability to produce high levels of active transforming growth factor (TGF)- β . (A) CMC-1 (SCC; 2×10^6) was inoculated intradermally into BALB/c mice (day 0). Anti-TGF- β mAb (500 μ g/mL) was i.p. injected at days 0, 3, 6, and 9. At day 10, we evaluated the number of Tregs by FACSCalibur. The number was calculated as follows: total cell number \times rate of Tregs among total cells. The data shown are the mean \pm SD of three mice. Similar results were obtained in three separate experiments. (B) Draining lymph nodes of mice treated with anti-TGF- β or control IgG mAb were immunostained with anti-CD4 mAb and anti-Foxp3 mAb 13 days after tumor inoculation. (C) TGF- β activities in the culture supernatants of six kinds of sarcoma lines and eight kinds of SCC lines were determined by growth inhibition assay of Mv1Lu cells. We defined the inhibition rate (%) as an inhibition index (II) of active TGF- β . Similar results were obtained in three separate experiments. * $P < 0.006$ between the two indicated groups.

CD25 mAb control IgG of 1D11 (blue triangle) showed a slight decrease in tumor growth compared with control IgG of PC61 + control IgG of 1D11 (white circle), but no mice were completely cured from tumor. However, when mice were treated with anti-TGF- β mAb combined with anti-CD25 mAb, a complete rejection of SCC was induced (red circle). These results indicate that blocking the recovery of Tregs by anti-TGF- β mAb during Treg-depletion therapy led to the complete cure of carcinoma-bearing mice.

Discussion

In this work, we show the distinct regulation of the immunosuppressive mechanism mediated by Tregs between MCA-induced carcinoma and sarcoma. Although sarcomas are rejected by

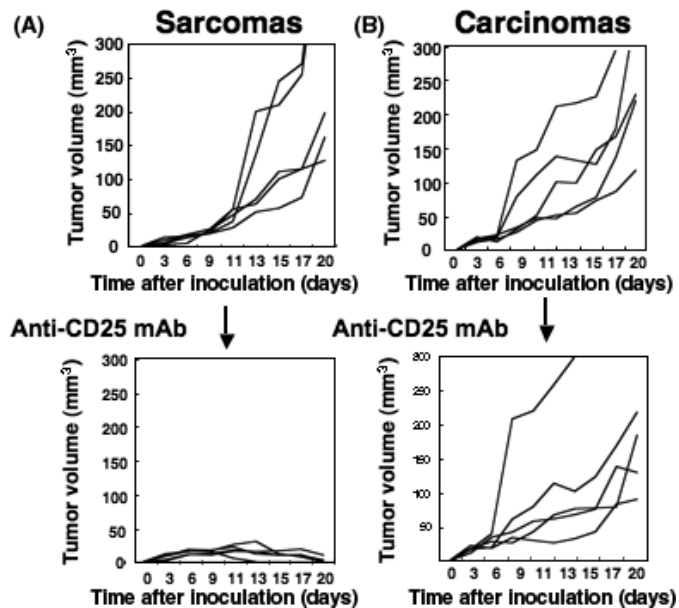


Fig. 4. 3-Methylcholanthrene-induced carcinomas, not sarcomas, are resistant to anti-CD25 mAb treatment. Five kinds of primary sarcoma (A) or squamous cell carcinoma (B) cell lines (2×10^6) were intradermally inoculated into BALB/c mice pre-treated with control mAb or anti-CD25 mAb (50 μ g/mouse). Effects of the regulatory T cell depletion were evaluated by monitoring subsequent tumor growth. Similar results were obtained in two separate experiments.

Treg-depletion therapy, SCCs are resistant to anti-CD25 mAb therapy because Tregs are rapidly recovered in DLN of SCC-bearing mice by highly produced, active TGF- β by SCC. However, combination treatment of SCC-bearing mice with anti-CD25 mAb (Treg-depletion) and anti-TGF- β mAb (Treg-recovery blockade) results in a complete rejection of SCCs.

It has been an important issue to resolve the mechanisms through which tumors exert their fatal growth in hosts.^(31,32) Recent published works have indicated that tumors can escape from immune surveillance mechanisms by inducing suppressive immunoregulatory cells, such as Tregs and myeloid-derived suppressor cells.^(3,5,7,8,14-22) The critical role of Tregs, in particular, for immunosuppression of antitumor immunity has been shown by the Sakaguchi group of researchers.^(15-18,24) They indicated that anti-CD25 mAb caused the depletion of Tregs and, in turn, activated host antitumor immunity to reject the established tumor.⁽¹⁶⁾ They also indicated that attenuating the effect of Tregs by targeting for GITR or CTLA-4 enhanced the efficacy of antitumor activity even in mice bearing large-established tumor.^(17,18) However, it has also been reported that natural killer (NK) cell-sensitive RL-male 1 and MethA sarcoma were completely rejected by Treg-depletion therapy, whereas B16 melanoma, colon carcinoma, and lung carcinoma cells were refractory to the treatment, although slight growth inhibition was observed.^(15,16,27,28) It remains poorly understood why some tumors are resistant to Treg-depletion therapy.

As shown in Figure 4, we initially found that there is a clear difference in the sensitivity against Treg-depletion therapy between primary SCC and sarcomas, induced by a single injection of MCA.⁽¹⁵⁾ We consider that it is very valuable to evaluate the reason why mouse carcinomas are more refractory to Treg-depletion therapy than sarcomas, in order to develop an efficient strategy to treat human cancers, most of which are carcinomas, not sarcomas.

In MCA-induced SCC-bearing mice, the number of Foxp3⁺ CD4⁺ T cells was increased in DLN, but neither spleen nor

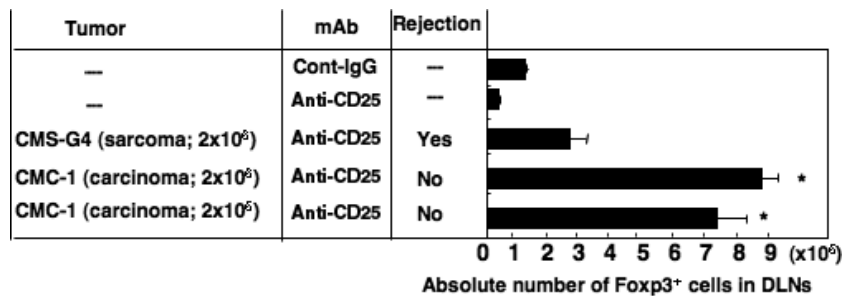


Fig. 5. Number of regulatory T cells rapidly restored in non-rejected tumor-bearing mice treated with anti-CD25 mAb. CMS-G4 (sarcoma; 2×10^6) and CMC-1 (carcinoma; 2×10^6 or 2×10^7) cells were intradermally inoculated into mice pre-treated with anti-CD25 mAb. Seven days after tumor inoculation, we evaluated absolute numbers of CD4⁺ Foxp3⁺ T cells in draining lymph nodes (DLNs) of the tumor-bearing mice by flow cytometric analysis. As controls (Cont), we analyzed tumor-free mice treated with control IgG or anti-CD25 mAb. The data shown are the mean \pm SD of four mice. Similar results were obtained in three separate experiments. —, tumor free or not applicable. * $P < 0.03$ versus CMS-G4-bearing mice group.

distal LN (Fig. 1). In tumor site, Foxp3⁺ T cells existed, as shown in Figure S1, but it was not easy to compare with LN and spleen simply because they were scattered in low density. As there was no difference in the immunosuppressive ability of Tregs in DLN, distal LN, and spleen (Fig. S1), the number of Tregs might be essential for assessing the inhibition they mediate. In previous papers,^(33,34) we showed that tumor-vaccination combined with CpG adjuvant or Th1 cells caused a great accumulation of tumor-specific CTL in DLN, but not in distal LN or spleen. Therefore, not only positive but also negative immunoregulatory cells appear to be activated at DLN of tumor-bearing mice. Indeed, we confirmed that Tregs were actively divided in DLN by Ki-67 staining (data not shown). Thus, the vigorous increase in Treg numbers in DLN of tumor-bearing mice might be rational for supporting tumor escape from antitumor immunity. The great increase of Tregs in DLN of SCC-bearing mice, but not sarcoma-bearing mice (Fig. 2), indicates that SCCs have stronger immunosuppressive activity than sarcomas through their profound Treg induction.

The numbers of Tregs in DLN of SCC-bearing mice were higher than those of sarcoma-bearing mice even when the mice were treated with anti-CD25 mAb to deplete Tregs before inoculation of tumors (Fig. 5). Moreover, Treg-depletion therapy induces the rejection of sarcomas but not SCCs (Fig. 4). Judging from these results, we hypothesized that TGF- β , produced at higher levels by SCC than sarcoma, might influence the rapid recovery of Tregs during tumor growth in Treg-depleted mice. As expected, it was shown that the refractoriness of SCC-bearing mice was overcome by the combination of anti-TGF- β mAb and anti-CD25 mAb (Fig. 6). By this combined treatment, the replenishment of Tregs was blocked, resulting in a complete cure of mice from SCC, which was refractory to anti-CD25 mAb treatment alone (Table 1 and Fig. 6). Although we suc-

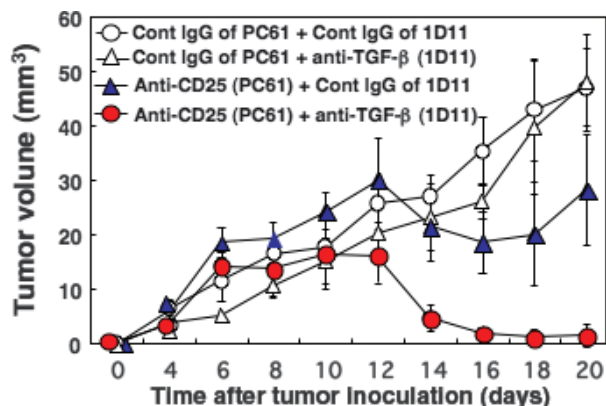
Table 1. Anti-transforming growth factor (TGF)- β mAb treatment inhibited the recovery of regulatory T cells (Tregs) after anti-CD25 mAb treatment

Tumor	Day 5			
	—	—	+	+
Anti-CD25	—	+	+	+
Anti-TGF- β	—	—	—	+
No. of Tregs ($\times 10^5$)	1.6 \pm 0.4	0.4 \pm 0.1	1.9 \pm 0.3*	1.1 \pm 0.1*

CMC-1 carcinoma cells (2×10^5) were inoculated into BALB/c mice. The number of Tregs in draining lymph nodes was counted 5 days after tumor inoculation (* $P < 0.03$). Unit, number of Tregs \pm SD ($n = 5$).

ceeded in the complete rejection of CMC-1, which produces high levels of TGF- β , by combination therapy of anti-CD25 mAb and anti-TGF- β mAb, it remains to be addressed whether the same strategy might be applicable to other carcinomas.

In this work, we showed that TGF- β -producing SCC were resistant to Treg-depletion therapy because of rapid Treg recovery mediated by TGF- β derived from SCC (Figs. 4–6). However, despite the result that some sarcoma lines produced the same level of TGF- β as carcinomas (Fig. 3C), all sarcomas were rejected by Treg-depletion therapy. To explain the contradiction, we further sought to investigate whether other mechanisms, such as NK/CTL sensitivity, immunosuppression mediated by M ϕ /CD11b⁺Gr-1⁺ cells, IL-10, and IL-6, were involved in the phenomenon. We did obtain evidence of higher NK sensitivity in sarcomas than SCCs (data not shown). Importantly, in some



Anti-CD25 mAb — + — +
Anti-TGF- β mAb — — + +

Fig. 6. Inhibiting regulatory T cell recovery by treatment with anti-transforming growth factor (TGF)- β mAb leads to complete rejection of carcinoma. CMC-1 (2×10^5) cells were inoculated at day 0 into BALB/c mice pre-treated with control IgG of PC61 (open circle, open triangle) or anti-CD25 mAb (PC61; filled circle, filled triangle). Anti-TGF- β mAb (1D11; open triangle, filled circle) or control IgG of 1D11 (open circle, filled triangle) was injected i.p. six times at 2-day intervals. Effects of the anti-CD25 mAb and anti-TGF- β treatment were evaluated by monitoring subsequent tumor growth. The data shown are the mean \pm SD of four mice. Photographs are representative at day 14. Similar results were obtained in three separate experiments.

published reports,^(16,35,36) NK cells were suppressed by Tregs as well as CTL, and were partly responsible for the rejection of some kinds of tumor when Tregs were depleted by anti-CD25 mAb.

The final goal of tumor immunotherapy is to induce fully activated tumor-specific CTLs in tumor-bearers. In previous papers,^(1,33) we showed that Th1 cell therapy is critical to overcome immunosuppressive tumor escape and induce antitumor immunity *in vivo*. The elimination of Treg from peripheral blood mononuclear lymphocytes caused the augmented induction of NY-ESO-1-specific Th1 cells, which enhance the generation of tumor-specific antitumor immunity.⁽³⁷⁾ It has been shown that tumor antigen-specific Tregs played a critical role for the primary mouse tumor system and for human tumor systems.^(38,39) Therefore, the control of tumor-specific Tregs will become important to induce a complete cure of tumor-bearers accompanied with the induction of antitumor immunity in tumor-bearing hosts. Here, we indicate that combination treatment of carcinoma-bearing mice with Treg-depletion therapy and Treg-recov-

ery blockade using anti-CD25 mAb and anti-TGF- β mAb, respectively, is an efficient strategy to induce a strong antitumor immunity and a complete rejection of tumor. We have already developed Th1 cell therapy, which can accelerate the generation of tumor-specific CTLs concomitantly with blocking the accumulation of Tregs in DLN of tumor-bearing mice.⁽⁴⁰⁾ We are now exerting the experiment by combination of the Th1 cell therapy with Treg - depletion and anti-TGF- β mAb treatment.

Acknowledgments

We thank Takuko Sawada (Shionogi Pharmaceutical Institute, Osaka, Japan) for the kind donation of IL-2. This work was partly supported by a Grant-in-Aid for National Project 'Knowledge Cluster Initiative' (2nd stage, Sapporo Biocluster Bio-S) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, a Grant-in-Aid for Scientific Research on Priority Areas, and a Grant-in-Aid for Young Scientists (B) from MEXT, the Akiyama Foundation, and the Osaka Cancer Research Foundation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Functional assay of regulatory T cells derived from tumor in draining lymph node (DLN), distal LN, and spleen.

Fig. S2. Ratio of regulatory T cells analyzed by immunochemical staining (green, Foxp3; red, CD4).

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