3-methylcholanthrene-induced TGF-β-producing carcinomas, but not sarcomas are refractory to Treg-depletion therapy

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Abstract

Regulatory T cell (Treg) is one of the major immunosuppressors in

tumor-bearing hosts. Although Treg-depletion therapy has been shown to induce a complete cure of tumor-bearing mice, this is not always successful treatment. Using 3-methylcholanthrene (MCA)-induced primary mouse tumors, we examined the distinct regulation of Treg-mediated immunosuppression between carcinomas and sarcomas. We demonstrated that the numbers of Tregs were greatly increased in SCC-bearing mice compared with sarcoma-bearing mice. This appeared to be because SCC produced higher levels of active TGF-β, which is essential for inducing Tregs, compared with sarcoma. Moreover, SCC, but not sarcomas were refractory to Treg-depletion therapy by anti-CD25 mAb administration. 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 of Treg recovery and induced a complete cure of SCC-bearing mice. Thus, we first demonstrated the refractoriness of mouse carcinoma against Treg-depletion therapy using anti-CD25 mAb administration. 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.

Introduction

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). Secondly, immunosupressions in tumor bearers should be removed to induce fully activation of tumor-specific CTLs (1-5). Several immunosuppressive mechanisms in tumor bearers have evaluated in animal experiments (2, 3, 5-10). However, the evaluated mechanisms in mice were not always applicable to clinical study (11, 12). The difficulties are because (i) many investigators used long-term cultured mouse tumor-cell lines, possessing distinct properties from primary tumors; (ii) most primary mouse tumors used for experiments are MCA-induced sarcoma even though over 98% of human cancer consisted 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 work (13), we have first established a novel carcinogenesis model to induce both squamous cell carcinoma (SCC) and fibrosarcoma by a single intradermally (i.d) injection of MCA. Using this method, we could easily and shortly 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.

Treg-mediated immunosupression is one of the major obstacles to cure 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, 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 immunosupression, this method did 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 anti-tumor defense mechanisms.

Here, we initially demonstrated that MCA-induced primary carcinomas were refractory to Treg-depletion therapy, while MCA-induced primary sarcomas were sensitive to it. This is because Tregs were rapidly recovered in carcinoma-bearing mice by active TGF-β, which was highly produced by carcinoma compared with sarcoma. Based on these findings, we demonstrated that combination treatment of SCC-bearing mice with anti-CD25 mAb and anti-TGF-β mAb caused a complete cure of tumor-bearing mice via blocking the rapid recovery of Tregs. Thus, our paper initially indicated 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 Japan (Yokohama, Japan). All mice were female and were used at 6-8 weeks of age. Squamous cell carcinomas (SCCs) and fibrosarcomas were obtained as described in previous paper (13). In brief, we injected 500μg of MCA intradermally. When the tumor reached >100 mm^3, we minced the tumor and seeded to 10cm dish. The cells were subcultured when they became confluent. We characterized cytokeratin-positive CMC-1 and CMC-9 as SCC, and vimentic-positive CMS-G2 and CMS-G4 as sarcoma by immunohistochmistry (13). We cultured SCCs and sarcomas in RPMI medium (1640, Sigma-Aldrich, Japan) containing 10% FCS (Nichirei Bioscience, Tokyo, Japan) and used within 10th passage. Mv1Lu cell (mink lung epithelial cell line) was donated by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Japan) and were cultured in MEME medium (M4655, Sigma-Aldrich, Japan) containing 10% FCS.

Reagents. Anti-Foxp3 polyclonal Ab for immunochemical staining was kindly donated by Dr. K Matsushima (Tokyo University, Japan) (29). Anti-Foxp3 mAb (150D/E4) used along with anti-Ki67 mAb and anti-Foxp3 mAb (FJK-16S) used along with anti-CD4 mAb were purchased from eBioscience (CA, USA). FITC-CD4 mAb (GK1.5) was purchased from DB bioscience (Fukushima,

Japan). Anti-Ki-67 mAb (TEC-3) was purchased from Dako Japan (Tokyo, Japan) and its control IgG mAb was purchased from DB bioscience. 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 (ATCC, VA, USA). Control IgG mAb of PC61 was purchased from DB bioscience. Recombinant human TGF-β1 (rhTGF-β) was purchased from PeproTech. rhIL-2 was kindly donated by T Yamada (SHIONOGI & CO., LTD. Osaka, Japan).

Immunochemical staining. Snap-frozen, Tissue-Tek-embedded lymph nodes were cut at 5-7μm, fixed for 10min 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 antibody against murine Foxp3 at a concentration of 5 mg/mL and anti- CD4 mAb (diluted 1 : 500). The secondary antibody was applied at a 1 : 500 dilution (anti-rat IgG-Alexa 546, anti-rabbit IgG-Alexa 488; Alexa, Leiden, The Netherlands). Finally, the samples were analyzed with 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

4hr 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 modified method described in previous paper (30). Concretely, Mv1Lu ($5x10^3$) were scattered in 96-well flat plate (Nunc, Roskilde, Denmark) in 100μl EMEM. We removed 50μl of the supernatant 12hr later and added 50μl of diluted-tumor supernatants. Then, 24hr later Mv1Lu growth inhibition was evaluated in ^{3}[H]-thymidine incorporation assay. We defined the inhibition rate (%) as an inhibition index (II) of active TGF-β. Inhibitin rate was calculated as follows; Inhibiton rate (%)= 100 x [cpm without sup – cpm with sup] / [cpm without sup]. In preparing sup, tumor cells (2×10^5) were scattered in 12-well plate (Corning) and changed the supernatant 12hr later to fresh medium of 1 ml. The supernatants were collected 24hr later and used them 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 DLN, diatal LN and spleen cells were treated with Fixation/ Permeabilization buffer attached with Foxp3 staining set (eBioscience). Two hours later, they were washed with Permiabilizing Solution Buffer and stained with anti-Ki67 mAb (50μg/ml) for 20 min. Cells were washed with Permiabilizing

Solution Buffer and stained with anti-rat IgG-Alexa 488 for 20 min. And then cells were washed again with Permiabilizing Solution Buffer and stained with PE-conjugated anti-Foxp3 mAb (150D/E4). FITC-CD4/ PE-Foxp3 staining was exerted according the instruction. Data were acquired on a Becton Dickinson FACSCalibur (Becton Dickinson, NJ, USA). Data were analyzed using CellQuest software (Becton Dickinson).

Treg-depletion therapy model. Anti-CD25 mAb (50μg/ head; PC61) or control rat IgG was intravenously (i.v.) injected 24hr before tumor inoculation. Anti-TGF-β mAb (500μg/ head; 1D11) or control mouse IgG (13C4) was intraperitoneally (i.p.) administered simultaneously with tumor inoculation and other 5 times at 2 days intervals. The tumor cells ($2x10^6$ or $2x10^5$) were i.d. inoculated in the left flank. The axillary lymph node were collected and analyzed as a draining lymph node. Tumor volume was calculated by the following formula: tumor volume = 0.4 x length (mm) x [width (mm)] ^{2}.

Statistical analyses. Mean values and standard deviation were calculated for data from representative experiment and are shown in the figures. Significant differences in the results were determined by the Student's *t*-test. $p < 0.05$ was considered as significant in the present experiments.

Results

The 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 SCC cell line, termed as CMC-1 (Fig. 1A). We also confirmed the similar increase of Tregs in DLN, but not distal LN and spleen by immunohistochemical analysis (Fig.1B). In tumor tissue, Tregs were scattered in low density over the tumor area (Supplementary Fig. S1).

We further addressed whether Tregs existing in DLN, distal LN and spleen had the different ability 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 respectively, and measured the cell growth. As shown in supplementary Fig. S2, functional differences were not observed between the Tregs prepared from each organ.

SCC 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 for experiments within 10 passages. To investigate the different regulation of Treg-mediated immunosuppression between MCA-induce sarcoma and SCC, we examined the increase of Tregs in DLN of mice bearing carcinoma or sarcoma. Two weeks after the inoculation of $2x10^6$ cells of sarcoma cell lines (CMS-G2, CMS-G4) or SCC lines (CMC-1, CMC-9), the numbers of Tregs increased in DLN were immunohistochemically counted. Interestingly, mice bearing with 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, which was highly produced by SCC but not sarcoma

In previous work (20, 21), it was already demonstrated 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 exhibited Treg regulation activity in vivo (Fig. 3A and 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 4 times at 2 days-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). The similar results were also

demonstrated in an immunohistochemical 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 the augmented Treg induction in SCC-bearing mice might be due to their higher producing ability of active TGF-β compared with sarcoma. To demonstrate our working hypothesis, the producibility of active TGF-β from SCC and sarcoma were 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 exhibited higher ability to produce active TGF-β than sarcoma lines (Fig. 3C). These results indicated that 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.

MCA-induced carcinoma is 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 antitumor immunity in vivo, we depleted Tregs by the treatment with anti-CD25 mAb before inoculation of several SCC lines or sarcoma lines at $2x10^6$ cells/mouse. Surprisingly, the administration of anti-CD25 mAb showed no significant effect on the growth of SCCs, while 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 contrastive TGF-β production (Fig.3D) and almost the same growth rates in vitro and in vivo (data not shown). We counted the number of Tregs in CMS-G4 or CMC-1-bearing mice after treatment with anti-CD25 mAb. As shown in Fig. 5, tumor-free mice treated with anti-CD25 mAb caused a great reduction of 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 totally different from the case of sarcoma, mice inoculated with 2×10^6 or 2×10^5 CMC-1 cells, which could not be rejected, exhibited a rapid and great 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.

SCC-bearing mice resistant to Treg-depletion therapy were cured from tumor by combined treatment with anti-TGF-β mAb. The results of Fig. 5 suggested that SCC-derived TGF-β might accelerate the recovery of Tregs after the depletion, which might induce the refractoriness to anti-CD25 mAb treatment. To examine the critical role of TGF-β for the rapid recovery of Tregs, we administered anti-TGF-β mAb (1D11) into CMC-1-bearing mice after the 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 tumor inoculation. To examine the anti-tumor effect of the conbination therapy, we next evaluated the tumor growth of the mice treated with both mAb. As shown in Fig. 6, CMC-1-bearing mice treated with anti-CD25 mAb + control IgG of 1D11 (blue triangle) showed slightly decrease of 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 demonstrated that blocking the recovery of Tregs by anti-TGF-β mAb during Treg-depletion therapy led to the complete cure of carcioma-bearing mice.

Discussion

In this work, we demonstrate the distinct regulation of immunosuppressive mechanism mediated by Tregs between MCA-induced carcinoma and sarcoma. Although sarcomas are rejected by Treg-depletion therapy, SCCs are resistant to anti-CD25 mAb-administration 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 why tumors expressing tumor antigens exhibit their fatal growth in host (31, 32). Recent works have demonstrated 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). Especially, the critical role of Tregs for immunosuppression of antitumor immunity has demonstrated by Sakaguchi group (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 with a large-established tumor (17, 18). However, it has been also reported that NK-sensitive RL-male 1 and MethA sarcoma were completely rejected by Treg-depletion therapy, while B16 melanoma, colon carcinoma and

lung carcinoma cells were refractory to the treatment though slightly growth inhibition was observed (15, 16, 27, 28). It remains poorly understood why some tumors are resistant to Treg-depletion therapy.

As shown in Fig.4, here, we initially find that there is a clear difference in the sensitivity against Treg-depletion therapy between primary SCC and sarcomas, which are induced by a single injection of MCA (13). We consider that it is greatly valuable to evaluate the reason why mouse carcinomas are more refractory to Treg-depletion therapy than sarcomas for developing an efficient strategy to human cancer, most of which are consists of carcinomas but not sarcomas.

In MCA-induced SCC-bearing mice, the number of $Foxp3^+$ $CD4^+$ T cells was increased in DLN, but neither spleen nor distal LN (Fig. 1). In tumor site, $foxp3^+$ T cells existed as shown in supplementary data Fig. S1, but it was not easy to compare with LN and spleen simply because they were scattered in low density. Since there was no different immunosuppressive ability among the Tregs of DLN, distal LN and spleen (supplementary Fig. S1), the number of Tregs might be essential for assessing the inhibition mediated by Tregs. In previous paper (33, 34), we demonstrated that tumor-vaccination combined with CpG adjuvant or Th1 cells caused a great accumulation of tumor-specific CTL in DLN but not distal LN and spleen. Therefore, not only positive, but also negative immunoregulatory cells appears to be activated at DLN of tumor-bearing mice. Indeed, we confirmed that Tregs were actively divided in DLN by Ki-67 stainig (Data not shown). Thus, the vigorous increase of Tregs in DLN of tumor-bearing

mice may 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), indicating that SCCs exhibit stronger immunosuppressive activity than sarcoma via 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 SSCs (Fig.4). Judging from these results, we hypothesized that TGF-β higher produced by SCC than sarcoma may be influence on a rapid recovery of Tregs during tumor growing in Treg-depleted mice. As expected, it was demonstrated that the refractoriness of SCC-bearing mice were overcomed by combination treatment with anti-TGF-β mAb administration in addition to anti-CD25 mAb（Fig.6）. By this combination 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 succeeded in the complete rejection of high highly TGF-β-producing CMC-1 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 paper, we demonstrated that TGF-β-producing SCC were resistant to Treg-depletion therapy because of rapid Treg recovery mediated by TGF-β derived from SCC (Fig. 4-7). 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 ought to investigate whether other mechanisms, such as NK / CTL sensitivity, immunosuppression mediated by Mϕ / $CD11b^{+}Gr\text{-}1^{+}$ cell, IL-10 and IL-6, were involved in the phenomenon. Actually, we obtained the evidence of higher NK sensitivity of sarcomas than that of SCCs (data not shown). Importantly, in some reports (16, 35,36), NK cells were suppressed by Tregs as well as CTL, and were partly responsible for the rejection of some kind 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 paper (1, 33), we demonstrated 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-sspecific Th1 cells, which enhance the generation of tumor-specific antitumor immunity (37). It has been demonstrated that tumor-antigen-specific Tregs played a critical role for primary mouse tumor system and in huma 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-recovery

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). Therefore, we are now trying the combination treatment of tumor-bearing mice with Th1 cell therapy combined with Treg-depletion therapy and anti-TGF-β mAb administration.

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Figure Legends

Fig. 1 Tregs were increased in DLN, but not distal LN and spleen in SCC-bearing hosts. A, CMC-1 ($2x10^6$; an representative SCC) was i.d. inoculated. The numbers of $CD4^+$ $Foxp3^+$ T cells in tumor-side axillary draining LN (DLN; filled circle), contralateral axillary LN (distal LN; open circle) and spleen (filled triangle) was analyzed 0, 4, 8, 12, 16 days after CMC-1 inoculation. The data are shown as mean±SD of three mice in each experimental group. Asterisk means significant difference between DLN and distal LN ($p < 0.05$). 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 described in Materials and Methods (red; CD4, green; Foxp3, x200). Representative data were shown in figures.

Fig. 2 The number of Tregs in DLN increases in MCA-induced SCC-bearing mice, but not sarcoma-bearing mice. A, BALB/c mice were i.d. inoculated with primary sarcoma lines (CMS-G2, CMS-G4; $2x10^6$) and primary SCC lines (CMC-1, CMC-9; $2x10^6$). DLN of the tumor-bearing mice were immunostained with anti-CD4 mAb (Alexa 546) and anti-Foxp3 mAb (Alexa 488) as Materials and Methods. Distributions of $CD4^+$ $Foxp3^+$ Tregs in the tumor DLN were evaluated by the immunohistochemical observation. B, The numbers of $Foxp3^+$ Tregs of the sections were counted. The data are shown as mean±SD of

5 fields of each DLN. Asterisk means significant difference between sarcoma and SCC ($p < 0.02$).

Fig. 3 SCCs predominantly induce Tregs via high producing ability of active TGF-β. A, CMC-1 (SCC; 2 x 10^6) was i.d. inoculated into BALB/c mice (day0). Anti-TGF-β mAb (500 μg/ml) was i.p. injected at day 0, 3, 6, 9. At day10, we evaluated the number of Tregs by FACSCalibur. The number was calculated as follows; total cell number x the rate of Tregs among total cells. The datas were shown as mean±SD of 3 mice. Similar results were obtained in three separate experiments. B, DLNs of the mice treated with anti-TGF-β or control IgG mAb were immunostained with anti-CD4 mAb (Alexa 546) and anti-Foxp3 mAb (Alexa 488) 13 days after tumor inoculation. C, TGF-β activities in the culture supernatants of 6 kinds of sarcoma lines and 8 kinds of SCC lines were determined by growth inhibition assay of Mv1Lu cells as descrived in Materials and Methods. We defined the inhibition rate (%) as an inhibition index (II) of active TGF-β. Asterisk means significant difference between the indicated two groups ($p < 0.006$). Similar results were obtained in three separate experiments.

Fig. 4 MCA-induced carcinomas, not sarcomas are resistant to anti-CD25 mAb treatment A-B, five kinds of primary sarcoma (A) or SCC (B) lines (2x10^6) were i.d. inoculated to BALB/c mice pre-treated with control mAb or anti-CD25 mAb (50 μg/mouse). Effects of the Treg depletion were evaluated by

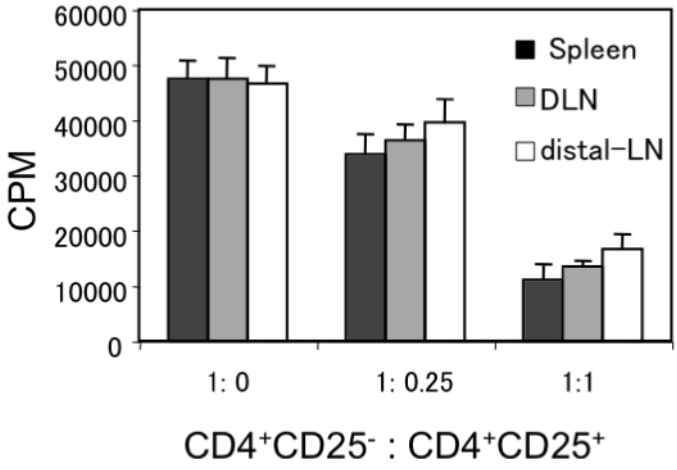
monitoring for the subsequent tumor growth. Similar results were obtained in two separate experiments.

Fig. 5 The number of Tregs rapidly restored in non-rejected tumor-bearing mice treated with anti-CD25 mAb. CMS-G4 (sarcoma; $2x10^6$) and CMC-1 (carcinoma; $2x10^6$ or $2x10^5$) were i.d. inoculated into the mice pre-treated with anti-CD25 mAb. Seven days after tumor inoculation, we evaluated absolute numbers of $CD4^+$ $Foxp3^+$ T cells in DLN of the tumor-bearing mice by flow cytometric analysis. As controls, we analyze tumor-free mice treated with control IgG or anti-CD25 mAb. The daras are shown as mean±SD of 4 mice. Asterisk means significant difference from CMS-G4-bearing mice group ($p < 0.03$). Similar results were obtained in three separate experiments.

Fig. 6 Inhibiting Treg recovery by administration of anti-TGF-β mAb lead to complete rejection of carcinma. CMC-1 (2×10^5) was 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 i.p. administered 6 times at 2 days intervals. Effects of the anti-CD25 mAb- and anti-TGF-β-treatment were evaluated by monitoring for the subsequent tumor growth. The datas are shown as mean±SD of 4 mice. Photos were the representative data at day14. Similar results were obtained in three

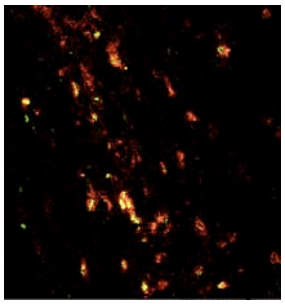
separate experiments.

Supplementary data of Fig.S1



Fnctional assay of Tregs derived from tumor- DLN, distal LN, spleen .
$CD4^+$ $CD25^-$ $CD45RB^+$ naïve T cells and $CD4^+$ $CD25^+$ T cells were isolated from nylon-passed spleen cells of BALB/c mice using FACS Aria (Becton Dickinson, San Jose, CA). They were stimulated with anti-CD3 mAb (0.5mg/ml, BD) at the ratio of in the presence of mitomycin C (MMC)-treated spleen cells ($5x10^4$) at 96well U plate (FALCON, NJ, USA) (Naïve T cell: Treg cell = 1:0, 1: 0.25, 1:2) . The proliferation was examined 24hr later in ^{3}[H]-thymidine incorporation assay as described in previous paper (1).

Supplementary data Fig.S2



The ratio of Tregs (CD4;red, Foxp3; green) were analyzed by immunochemically staining. Tregs were detected in tumor site by immunohistochemical analysis described in Materials and Methods (red; CD4, green; Foxp3, x200). Representative data was shown in figure.

Table 1.
Anti-TGF-β mAb treatment inhibited the recovery of Tregs after anti-CD25 mAb treatment.

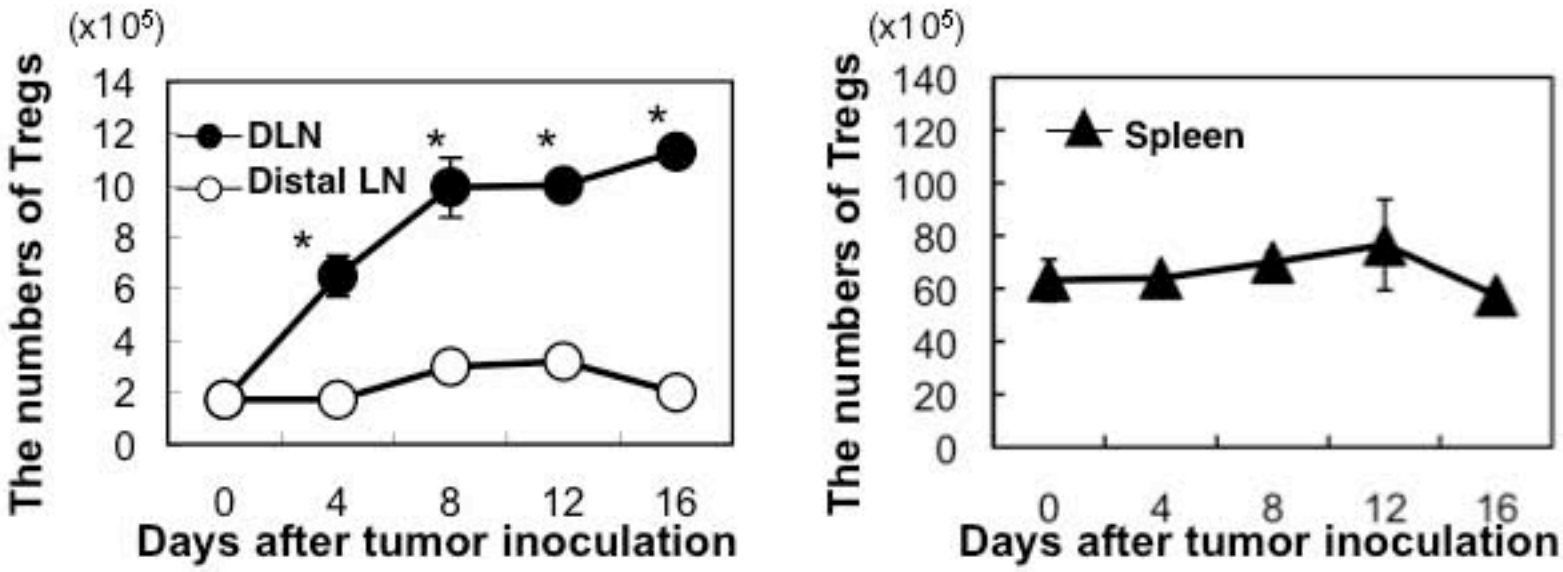
	Day5			
Tumor	-		+	
Anti-CD25	-	+	+	+
Anti-TGF-β	-	-	-	+
Number of Treg ($x10^5$)	**1.6 ±0.4**	**0.4 ±0.1**	**1.9 ±0.3**	**1.1 ±0.1**

*

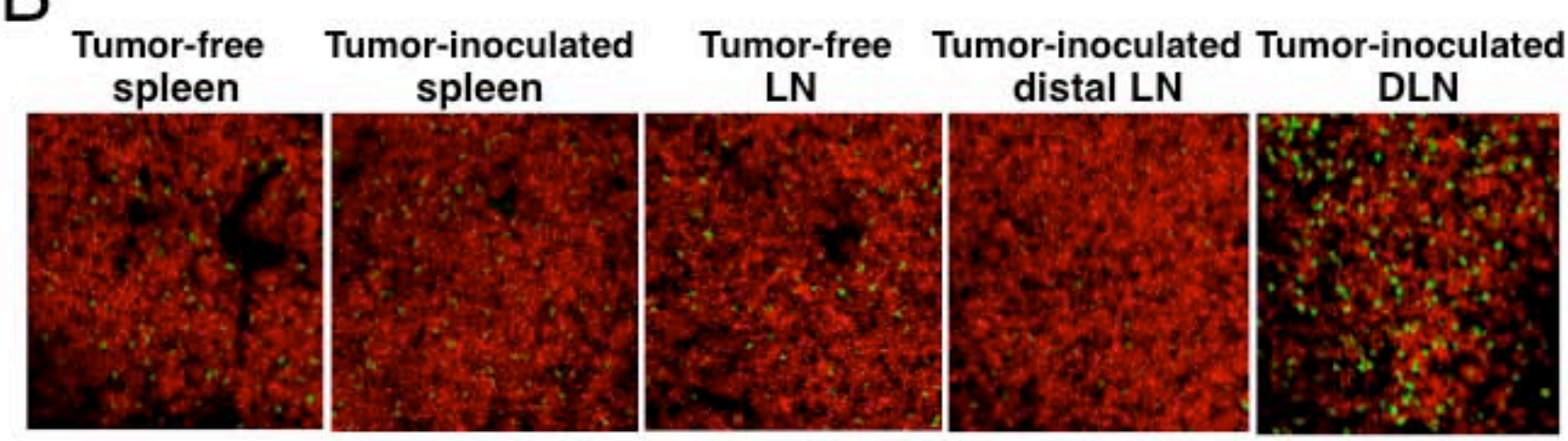
$2x10^5$ CMC-1cells were inoculated. The number of Tregs in DLN were counted 5 days after tumor inoculation (* $p<0.03$).
Unit; The number of Tregs ± S.D. (n=5)

Fig.1 Chamoto et al.

A



B



x200 Red:CD4, Green: Foxp3

Fig.2 Chamoto et al.

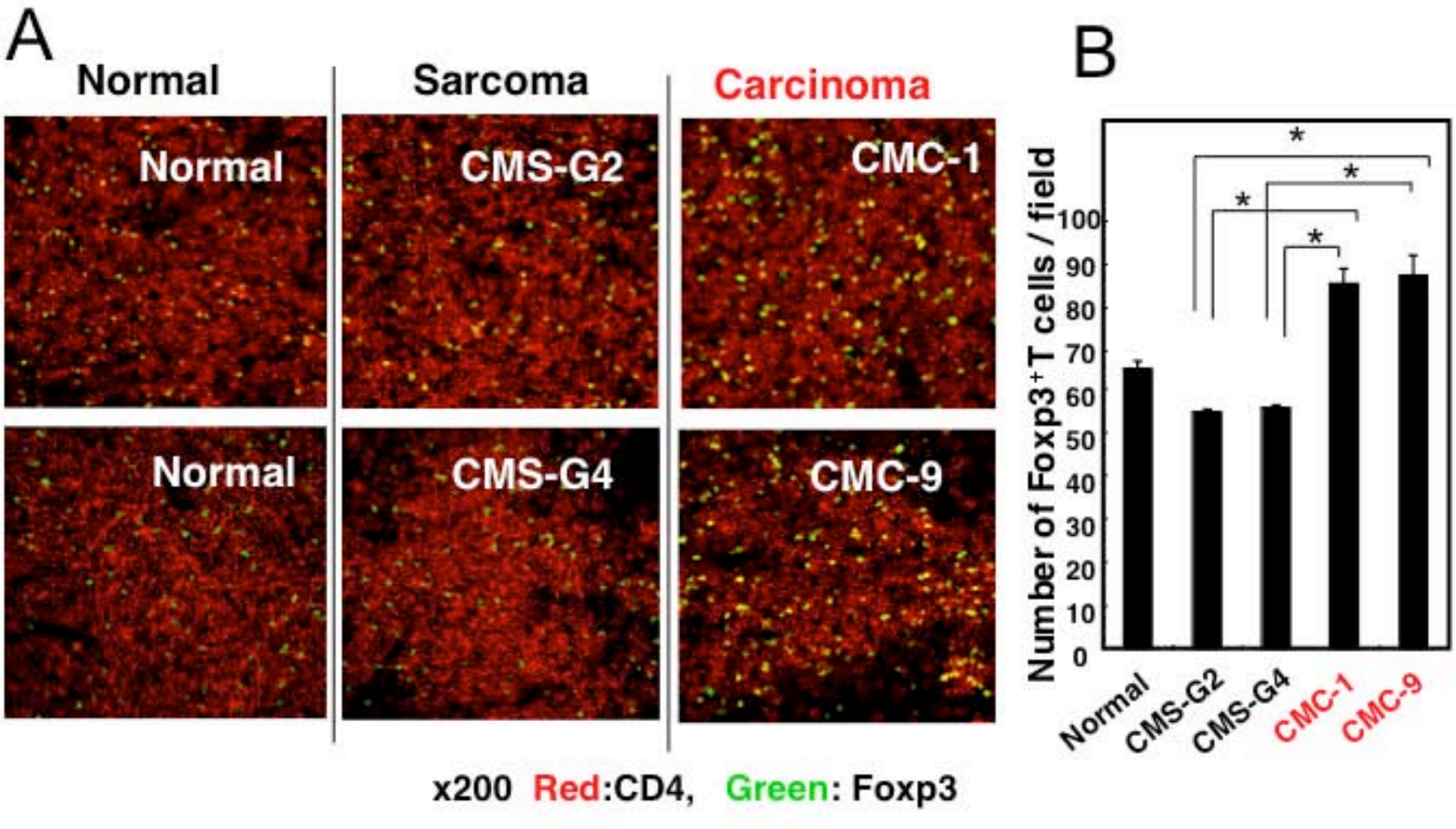
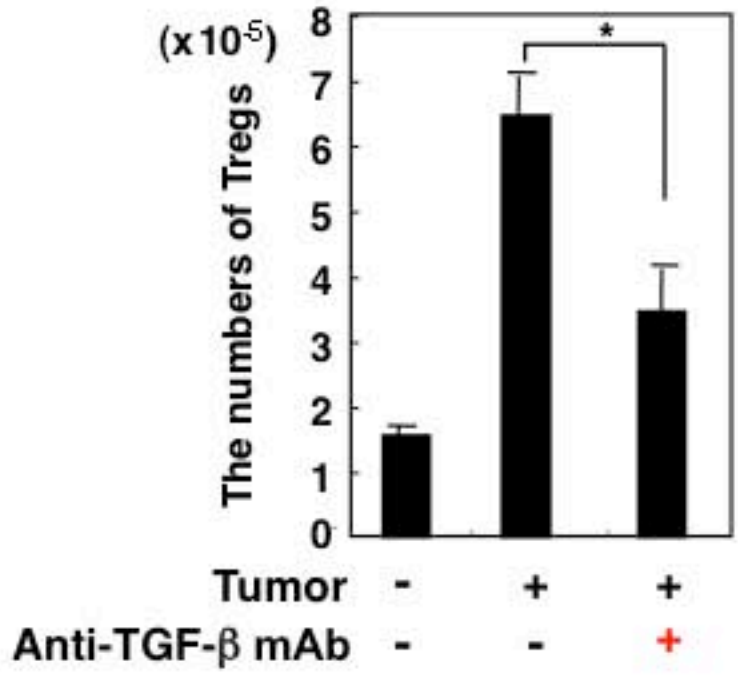


Fig.3 Chamoto et al.

A



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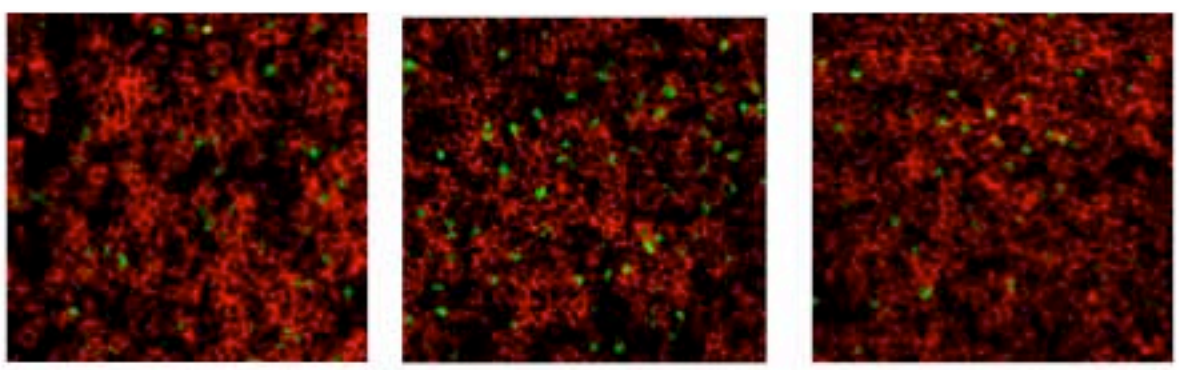

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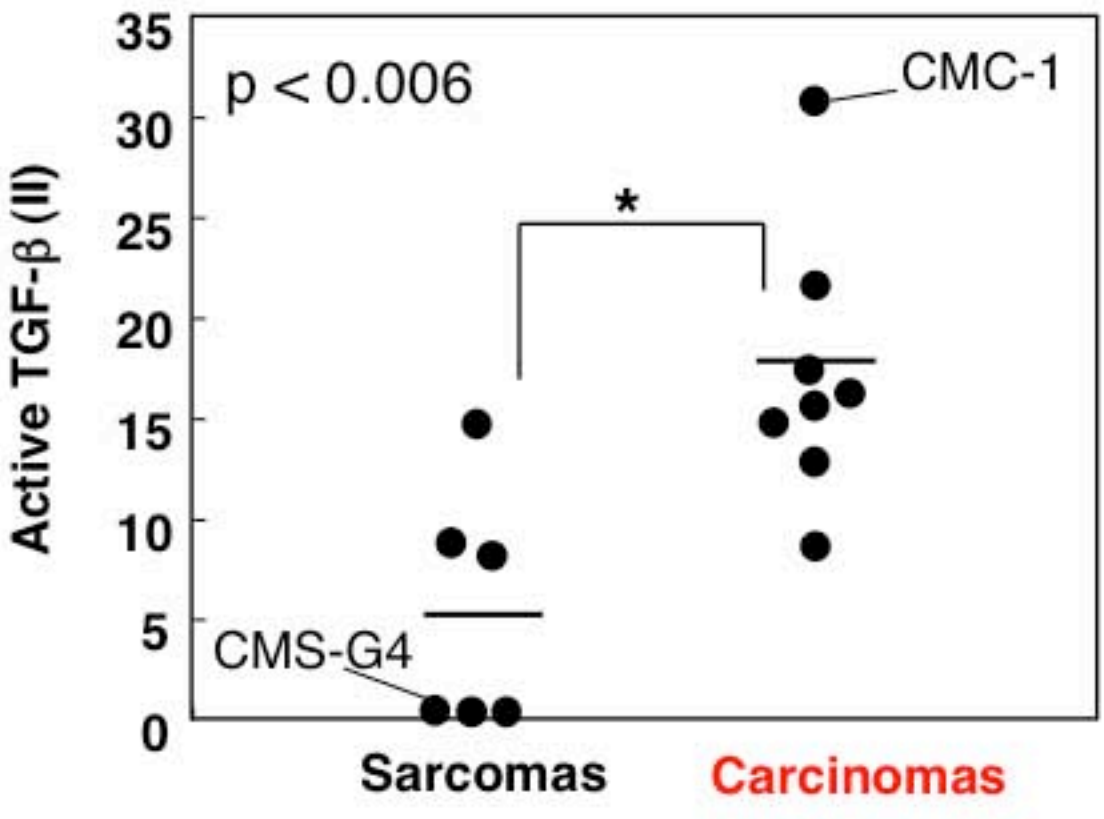

Fig.4 Chamoto et al.

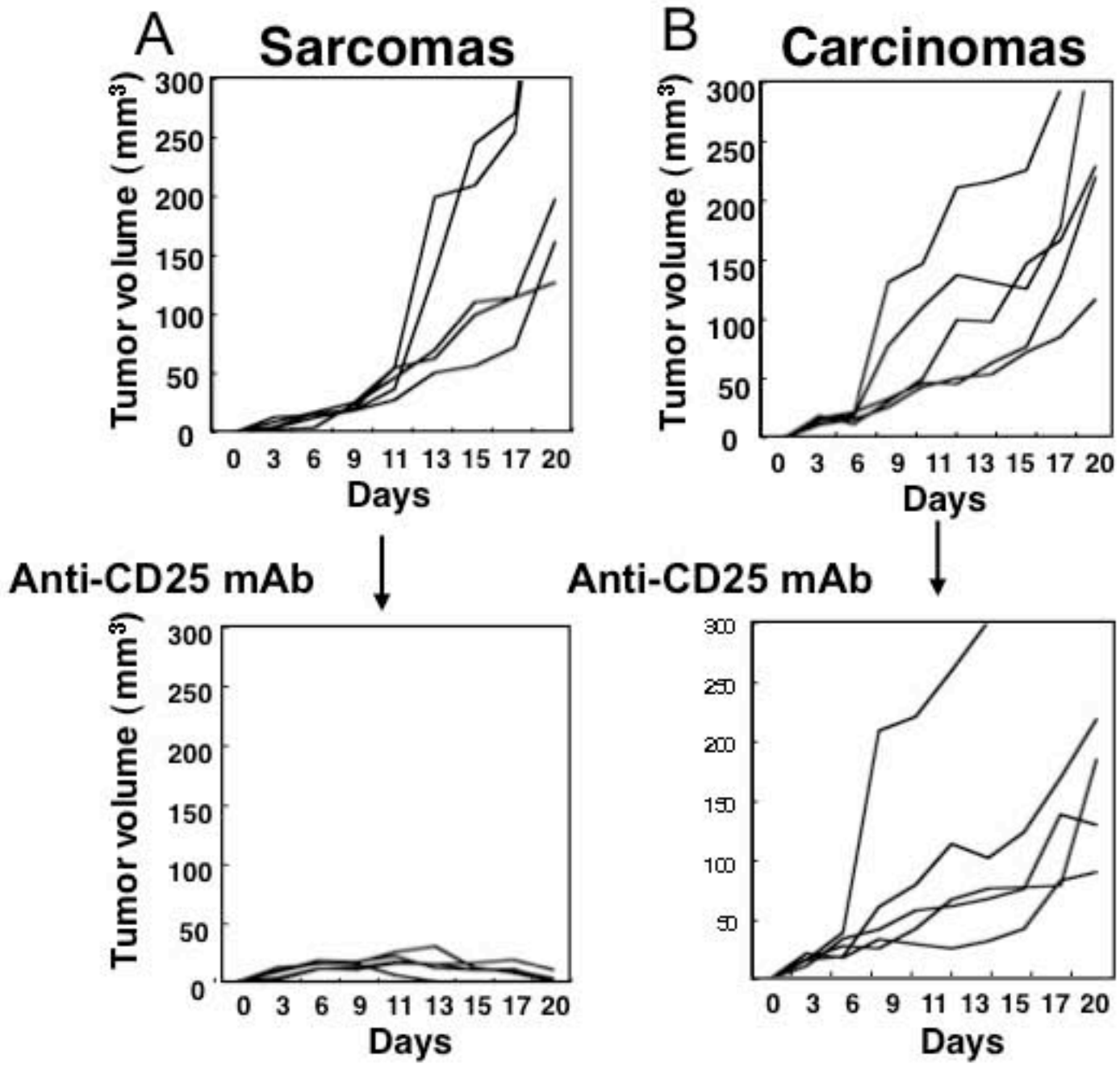

Fig.5 Chamoto et al.

Tumor	mAb	Rejection
-	Cont-IgG	-
-	anti-CD25	-
CMS-G4 (sarcoma; $2x10^6$)	anti-CD25	Yes
CMC-1 (carcinoma; $2x10^6$)	anti-CD25	No
CMC-1 (carcinoma; $2x10^5$)	anti-CD25	No

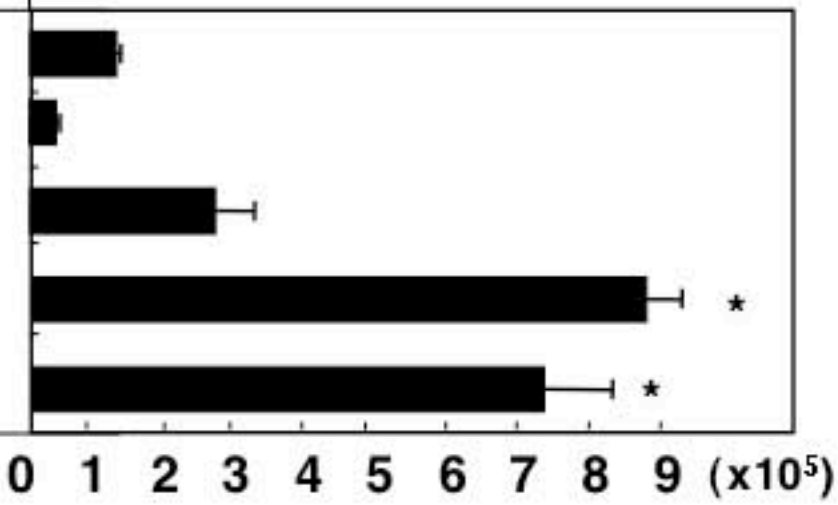

Fig.6 Chamoto et al.