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# Sequential Involvement of Two Distinct CD4$^+$ Regulatory T Cells during the Course of Transplantable Tumor Growth and Protection from 3-Methylcholanthrene-induced Tumorigenesis by CD25-depletion

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**The involvement of two phenotypically different regulatory T cells in different stages of tumor growth was investigated. Treatment of BALB/c mice with anti-CD25 monoclonal antibody (mAb) (PC61), but not anti-CD4 mAb (GK1.5) before RL male 1 or Meth A inoculation caused tumor rejection. On the other hand, treatment of BALB/c mice with anti-CD4 mAb (GK1.5) but not anti-CD25 mAb (PC61) on day 6 after inoculation of the same tumors caused rejection. The findings suggest that CD4$^+$CD25$^+$ T cells downregulated the rejection response in the early stage of tumor growth. On the other hand, putative CD4$^+$CD25$^-$ T cells downregulated the tumor rejection response in the late stage. Both CD4$^+$CD25$^+$ and putative CD4$^+$CD25$^-$ T cells appeared to inhibit the efficient generation of cytotoxic T lymphocytes (CTL). The present study also demonstrated that the treatment of BALB/c mice with anti-CD25 mAb (PC61) at 4 or 6 weeks after 3-methylcholanthrene (3-MC) inoculation retarded tumor occurrence and prolonged survival.**

Key words: Tumor growth — Regulatory T cells — CTL — Chemical carcinogenesis

CD4$^+$CD25$^+$ cells consist of ~10% of CD4$^+$ cells in the peripheral lymphoid tissues and were shown to be a unique population of immunoregulatory cells.[1–7] Sakaguchi and his colleagues demonstrated that transfer of BALB/c spleen cells depleted of CD25$^+$ cells into athymic BALB/c mice caused various autoimmune diseases which could be prevented by co-transfer of CD4$^+$CD25$^+$ cells.[2, 3] Prolonged *in vivo* depletion of CD25$^+$ cells by administration with anti-CD25 monoclonal antibody also caused autoimmunity.[8] CD4$^+$CD25$^+$ T cells were shown to be anergic to stimulation via T cell receptor (TCR), and they suppressed antigen-specific T cell proliferation when cultured with them.[6, 7] Furthermore, recently, it has been reported that the CD28/B7 costimulatory pathway is essential for the development and homeostasis of CD4$^+$CD25$^+$ regulatory T cells.[9] CD4$^+$CD25$^+$ T cells with a similar immunoregulatory function have been shown to be present also in humans.[10]

We previously demonstrated that *in vivo* administration of anti-CD4 monoclonal antibody (mAb) (GK1.5) on day 6 after tumor inoculation resulted in rejection of RL male 1 tumor which grew and killed untreated BALB/c mice. The treatment induced cytotoxic T lymphocyte (CTL) generation against a dominant antigen pRL1a on RL male 1.[11] Furthermore, we showed that *in vivo* administration of anti-CD25 mAb (PC61) before tumor inoculation caused rejection of some immunogenic tumors which grew and killed untreated syngeneic mice.[12] In this study, we extended those analyses and studied the relation of CD4$^+$ and CD4$^+$CD25$^+$ regulatory T cells in tumor growth. We showed that CD4$^+$CD25$^+$ and putative CD4$^+$CD25$^-$ regulatory cells were involved in early and late stages in tumor growth, respectively, and the occurrence of putative CD4$^+$CD25$^-$ regulatory cells was dependent on CD4$^+$CD25$^+$ cells. Furthermore, we studied the involvement of those regulatory T cells in tumorigenesis induced by 3-methylcholanthrene (3-MC).

## MATERIALS AND METHODS

**Mice** BALB/c mice were purchased from Japan SLC (Shizuoka). CB-17 SCID mice were provided by Dr. Kuribayashi (Mie University School of Medicine, Mie).

**Tumor cells** RL male 1 is a radiation-induced leukemia in a BALB/c mouse.[13] Meth A is a MC-induced fibrosarcoma in a BALB/c mouse.[14] These tumors were maintained by tissue culture and as ascites form. P1.HTR is a subline of DBA/2 MC-induced mastocytoma P815.[15]

**Antibodies** Anti-CD25 (IL-2R$\alpha$) mAb produced by hybridoma PC61 is a rat IgG1 antibody.[16] For *in vivo* administration, anti-CD25 mAb (PC61) was used after purification. The hybridoma ascites produced in CB-17 SCID mice was purified to homogeneity by ammonium sulfate precipitation, followed by chromatography on a DEAE Toyopearl 650S column (Tosoh, Tokyo). Anti-L3T4 (CD4) mAb produced by hybridoma GK1.5, provided by Dr. F. Fitch (University of Chicago, Chicago, IL), is a rat IgG2b antibody.[17] Anti-CD4 mAb (GK1.5) was used in the form of ascites.

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**Flow cytometry** Cells ($1\times10^6$) were incubated with mAb for 30 min at 4°C in 2% fetal calf serum (FCS) containing phosphate-buffered saline (PBS). Phycoerythrin (PE)-conjugated anti-CD3ε mAb (145-211), anti-CD4 mAb (GK1.5), anti-CD8α mAb (53-6.7) and FITC-conjugated anti-CD25 mAb (7D4) were used. These mAbs were purchased from PharMingen (San Diego, CA). After incubation with mAb, the cells were washed, and analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

***In vitro* generation of CTL** Spleen cells ($5\times10^7$) were cultured with $5\times10^6$ mitomycin C (MMC)-treated stimulator cells in tissue culture flasks (25 100, Corning Glass, Corning, NY) at 37°C in a 5% $CO_2$ atmosphere for 7 days. The cells were treated with MMC at a concentration of 50 μg/ml at 37°C for 30 min. The culture medium was RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μ*M* 2-ME (2-mercaptoethanol).

**Cell-mediated cytotoxicity assay** Tumor cells ($2\times10^6$) were labeled with 2 MBq of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) by incubation for 1.5 h at 37°C in a 5% $CO_2$ atmosphere. For peptide pulsing, $5\times10^{-6}$ *M* pRL1a peptide [18, 19] was added to $2\times10^6$ labeled P1.HTR cells. After incubation for 1 h, the cells were washed and used as target cells. In assays, $5\times10^3$ labeled target cells (100 μl) were incubated with effector cell suspension (100 μl). After incubation for 4 h at 37°C in a 5% $CO_2$ atmosphere, the supernatants (100 μl) were removed and their radioactivity was measured. The percentage of specific lysis was calculated as follows: $((a-c)/(c-b))\times100$, where *a* is the radioactivity of the supernatant from target cells mixed with effector cells, *b* is that of the supernatant from the target cells incubated alone, and *c* is that of the supernatant after lysis of the target cells with 1% Nonidet P-40.

**Antibody administration** The mice were anesthetized with ether, and mAb diluted in PBS (0.2 ml) was injected through the retrobulbar venous plexus.

**Induction of tumors by 3-MC** 3-MC (Sigma, St. Louis, MI) was dissolved in peanut oil at a concentration of 250 μg/ml and injected subcutaneously.

**Tumor assay** Tumor cells were injected intradermally into the mice with a 27-gauge needle. Before inoculation of the tumor cells, the hair was cut with clippers. The diameter of the tumors was measured with vernier calipers twice at right angles to calculate the mean diameter.

## RESULTS

**Effect of *in vivo* administration of anti-CD4 mAb (GK1.5) and anti-CD25 mAb (PC61) on RL male 1 and Meth A tumor growth in BALB/c mice** The effect of *in vivo* administration of anti-CD4 mAb (GK1.5) and anti-CD25 mAb (PC61) on tumor growth was investigated after inoculation of RL male 1 and Meth A into the back of BALB/c mice. As shown in Fig. 1, the administration of anti-CD4 mAb (GK1.5) on day 6 but not day −4 caused rejection of either RL male 1 or Meth A. On the other hand, the administration of anti-CD25 mAb (PC61) on day −4 but not day 6 resulted in rejection of the same tumors.

We further investigated the effect of anti-CD25 mAb (PC61) administered before or after tumor inoculation by sequential inoculation of different tumors at opposite flanks in a single mouse. BALB/c mice were inoculated with RL male 1 or Meth A as the first tumor, and treated

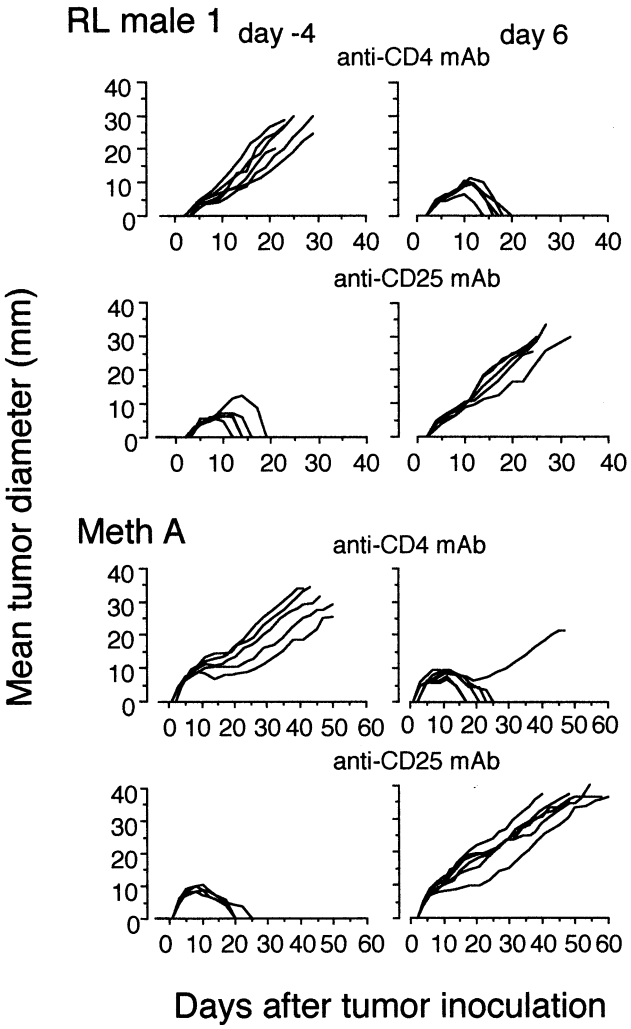


Fig. 1. Effect of *in vivo* administration of anti-CD4 mAb (GK1.5) and anti-CD25 mAb (PC61) on tumor growth before and after inoculation of RL male 1 and Meth A. BALB/c mice that had been inoculated on the back with tumor cells ($5\times10^5$) were treated with anti-CD4 mAb (GK1.5) (25 μl ascites diluted 1:8) or anti-CD25 mAb (PC61) (0.25 mg) on day −4 or day 6. Each line denotes tumor growth in an individual mouse.

with anti-CD25 mAb (PC61) on day 6, and then inoculated with Meth A or RL male 1, respectively, on day 10 as the second tumor, and the tumor growth was observed. As shown in Fig. 2, anti-CD25 mAb (PC61) had no effect on growth of the first tumor inoculated before the treatment with anti-CD25 mAb (PC61), and caused rejection of the second tumor inoculated after the treatment with anti-CD25 mAb (PC61).

**Depletion of $CD4^+CD25^+$ cells in tumor-bearing mice by treatment with anti-CD25 mAb (PC61)** Depletion of $CD4^+CD25^+$ cells in naive mice by *in vivo* administration of anti-CD25 mAb (PC61) was shown previously.[12] To confirm that $CD4^+CD25^+$ cells were also depleted in tumor-bearing mice in the presence of tumor antigen stimulation, RL male 1-bearing BALB/c mice were treated on day 6 with anti-CD25 mAb (PC61) and $CD25^+$ cells in the inguinal lymph node cells were determined on day 10 by flow cytometry. As shown in Fig. 3, approximately 75% reduction was observed in $CD4^+CD25^+$ cells. The results were essentially similar to those observed in naive mice. No significant reduction was observed with $CD8^+CD25^+$ cells.

**CTL generation in spleen cells from CD25-depleted, RL male 1-inoculated BALB/c mice** *In vivo* administration of anti-CD4 mAb (GK1.5) on day 6 after RL male 1 tumor inoculation caused rejection. Generation of a dominant antigen peptide pRL1a-specific CTL was shown in the spleen cells obtained from CD4-depleted, but not undepleted RL male 1-inoculated BALB/c mice.[11] *In vivo* depletion of $CD4^+CD25^+$ cells caused rejection of some immunogenic tumors which grew and killed the untreated mice (ref. 11) and this study). To investigate the mechanisms of the induction of the rejection response by CD25-depletion, we examined CTL generation in the spleen cells from CD25-depleted, RL male 1-inoculated BALB/c mice 6, 12, and 18 days after tumor inoculation. As shown in Fig. 4, vigorous CTL generation was observed in the

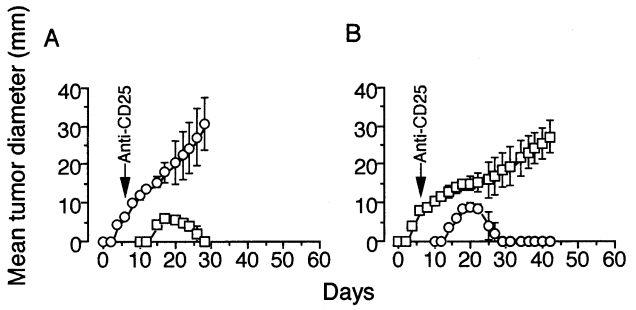


Fig. 2. Effect of *in vivo* administration of anti-CD25 mAb (PC61) on tumor growth before and after inoculation of RL male 1 and Meth A successively in a single mouse. BALB/c mice that were inoculated on the left flanks with the first tumors (RL male 1 in A, and Meth A in B) were treated with anti-CD25 mAb (PC61) (0.25 mg) on day 6. On day 10 after the first tumor inoculation, the mice were inoculated on the right flanks with the second tumors (Meth A in A, and RL male 1 in B) and the tumor growth was observed. Each group consisted of 10 mice. ○ RL male 1, □ Meth A.

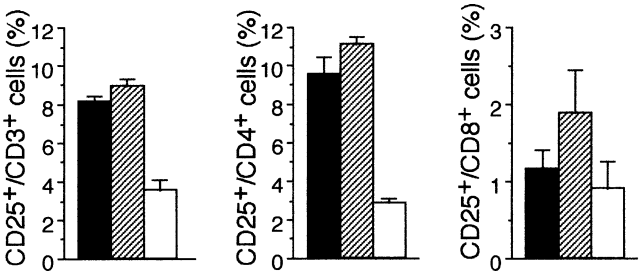


Fig. 3. Reduction of $CD4^+CD25^+$ T cells in tumor bearing mice by *in vivo* administration of anti-CD25 mAb (PC61) (0.25 mg). BALB/c mice that had been inoculated with RL male 1 ($5\times10^5$) were treated with anti-CD25 mAb (PC61) (0.25 mg) on day 6. Four days after treatment with mAb, lymph node cells from untreated (■), RL male 1-bearing (▨), or RL male 1-bearing mice treated with anti-CD25 mAb (□) were obtained and analyzed by FACScan. Each group consisted of 3 mice. Values indicate mean±SD.

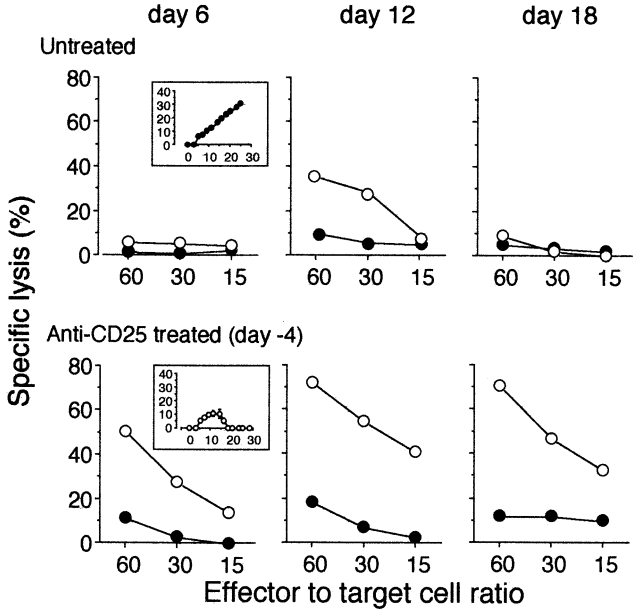


Fig. 4. Kinetic analysis of generation of CTL against a dominant pRL1a antigen in spleen cells from BALB/c mice that were inoculated with RL male 1 ($5\times10^5$), and either untreated or treated with anti-CD25 mAb (PC61) (0.25 mg) on day −4. Cytotoxicity was determined by 4-h $^{51}Cr$ release assay. Assays were done in duplicate. Target cells: ● P1.HTR, ○ pRL1a-pulsed P1.HTR. RL male 1 growth in mice untreated or treated with anti-CD25 mAb (PC61) is shown in the insets.

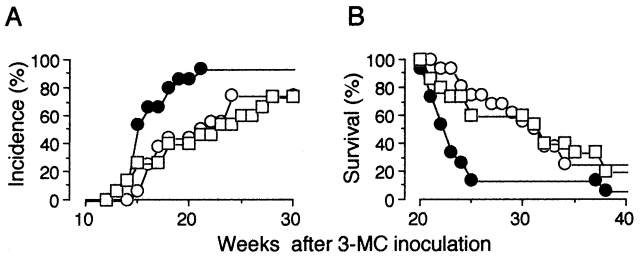


Fig. 5. Effect of *in vivo* administration of anti-CD25 mAb (PC61) on 3-MC-induced tumorigenesis. BALB/c mice that had been inoculated with 3-MC (25 $\mu$g) were left untreated (●) or treated with anti-CD25 mAb (PC61) (0.25 mg) at 4 (○) or 6 weeks (□) after inoculation of 3-MC. Mice were observed for tumor appearance and survival 2–3 times a week for more than 280 days. Each group consisted of 15 mice.

spleen cells from CD25-depleted, RL male 1-inoculated BALB/c mice. On the other hand, only transient CTL generation was observed on day 12 but not day 6 or day 18 in the spleen cells obtained from CD25-undepleted, RL male 1-inoculated BALB/c mice.

**Effect of *in vivo* administration of anti-CD25 mAb (PC61) on tumorigenesis by 3-MC** The effect of *in vivo* administration of anti-CD25 mAb (PC61) on tumorigenesis by 3-MC was investigated. BALB/c mice were inoculated s.c. with 3-MC (25 $\mu$g) into the back, and after 4 or 6 weeks, they were injected i.v. with anti-CD25 mAb (PC61). Palpable tumor appeared around 10 weeks after 3-MC inoculation in those mice. As shown in Fig. 5, retardation of tumor occurrence and prolongation of survival was observed.

## DISCUSSION

In this study, we showed that two phenotypically different regulatory T cells were involved in different stages of tumor growth in BALB/c mice. Treatment of BALB/c mice with anti-CD25 mAb (PC61), but not anti-CD4 mAb (GK1.5) before RL male 1 or Meth A inoculation caused tumor rejection. On the other hand, treatment of BALB/c mice with anti-CD4 mAb (GK1.5) but not anti-CD25 mAb (PC61) on day 6 after inoculation of the same tumors caused rejection. Treatment of the mice with anti-CD25 mAb (PC61) later than 2 days and with anti-CD4 mAb (GK1.5) earlier than 5 days after tumor inoculation showed no tumor rejection. The findings suggested that $CD4^+CD25^+$ T cells downregulated the rejection response in the early stage of tumor growth. On the other hand, putative $CD4^+CD25^-$ T cells downregulated the tumor rejection response in the late stage. Both $CD4^+CD25^+$ and putative $CD4^+CD25^-$ T cells appeared to inhibit the efficient generation of CTL, as was shown for $CD4^+CD25^+$ T cells in the present study and for putative $CD4^+CD25^-$ T cells in the previous study.[11] The finding that the treatment of mice with anti-CD25 mAb (PC61) before tumor inoculation did not result in continuous tumor growth also suggested that putative $CD4^+CD25^-$ regulatory T cells did not appear in the late stage of tumor growth following CD25-depletion. Thus, the generation of putative $CD4^+CD25^-$ regulatory T cells in the late stage of tumor growth was dependent on $CD4^+CD25^+$ regulatory T cells present in the early stage. Putative $CD4^+CD25^-$ regulatory T cells could be generated directly from $CD4^+CD25^+$ regulatory T cells by downregulating the CD25 expression. Alternatively, those two populations could be derived from independent lineages of T cell differentiation. Although putative $CD4^+CD25^-$ regulatory T cells appeared to be present in the late stage of tumor growth, those T cells may also be present in the early stage of tumor growth as a small population. In the early stage of tumor growth, $CD4^+$ helper T cells were necessary for generating effector T cells. Depletion of $CD4^+$ T cells by the administration of anti-CD4 mAb (GK1.5) before inoculation of the immunogenic tumors that were rejected in untreated mice abrogated the rejection response.[11] Treatment of the mice with anti-CD4 mAb (GK1.5) in the early stage of tumor growth depleted both $CD4^+$ helper T cells and $CD4^+$ regulatory T cells (if there were any), resulting in tumor growth. The experiment shown in Fig. 2 clearly indicated that putative $CD4^+CD25^-$ regulatory cells were specific for each tumor. North and Bursuker[20] previously showed that $CD4^+$ T cells from tumor-bearing mice specifically inhibited the effector T cell function in an adoptive transfer experiment. Although the specificity of $CD4^+CD25^+$ regulatory cells could not be analyzed in this study, studies by others showed that those cells recognized antigens specifically and exerted the inhibitory effect in a non-specific fashion.[6, 21] Stephens and Mason[22] recently showed that regulatory T cells in the peripheral lymphoid tissues that prevented autoimmune diabetes in rats were in the fractions of both $CD25^+$ and $CD25^-$ subpopulations. On the other hand, those T cells in the thymus were observed only in the fraction of $CD25^+$ T cells. Those findings are consistent with the present findings. In this study, we did not observe any autoimmune disease by histological examination 3 months after the anti-CD25 mAb (PC61) treatment. Taguchi and Takahashi[8] reported the occurrence of autoimmune disease after an *in vivo* administration of anti-CD25 mAb (PC61) by successive treatment over a prolonged time period (daily mAb injection for 11 days) and with a higher amount (1 mg per shot). The occurrence of autoimmune disease may be related to the severity of CD25-depletion.

The present study also demonstrated that the treatment of BALB/c mice with anti-CD25 mAb (PC61) at 4 or 6 weeks after MC inoculation retarded tumor occurrence and prolonged survival. Shankaran *et al.*[23] recently demon-

strated that lymphocytes and interferon (IFN) γ collaborate to protect against the development of carcinogen-induced sarcomas and spontaneous epithelial carcinoma and also to select for tumor cells with reduced immunogenicity, using immunodeficient mice that lacked the recombination-activating gene-2 (*RAG2*) and IFNγ-insensitive mice that lacked either the IFNγ receptor or STAT1 owing to gene disruption. The present findings suggested that $CD4^+CD25^+$ T cells were involved in such immunosurveillance against tumorigenesis induced by 3-MC.

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