Robust Antitumor Effects of Combined Anti-CD4-Depleting Antibody and Anti-PD-1/PD-L1 Immune Checkpoint Antibody Treatment in Mice

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Abstract

Depletion of CD4+ cells in tumor-bearing mice has strong antitumor effects. However, the mechanisms underlying these effects and the therapeutic benefits of CD4+ cell depletion relative to other immunotherapies have not been fully evaluated. Here, we investigated the antitumor effects of an anti-CD4-depleting mAb as a monotherapy or in combination with immune checkpoint mAbs. In B16F10, Colon 26, or Lewis lung carcinoma subcutaneous tumor models, administration of the anti-CD4 mAb alone had strong antitumor effects that were superior to those elicited by CD25+ Treg depletion or other immune checkpoint mAbs, and which were completely reversed by CD8+ cell depletion. CD4+ cell depletion led to the proliferation of tumor-specific CD8+ T cells in the draining lymph node and increased infiltration of PD-1+CD8+ T cells into the tumor, with a shift toward type I immunity within the tumor. Combination treatment with the anti-CD4 mAb and immune checkpoint mAbs, particularly anti–PD-1 or anti–PD-L1 mAbs, synergistically suppressed tumor growth and greatly prolonged survival. To our knowledge, this work represents the first report of robust synergy between anti-CD4 and anti–PD-1 or anti–PD-L1 mAb therapies. Cancer Immunol Res; 3(6); 631–40. ©2015 AACR.

Introduction

Immune checkpoint modulators such as those targeting cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) have attracted attention due to their extraordinary antitumor effects in patients with advanced melanoma, lung cancer, and renal cancer (1, 2). An mAb against CTLA-4 (ipilimumab) that enhances both early T-cell activation and CTL function was approved for treatment of patients with advanced melanoma in the United States in 2011. An anti–PD-1 mAb (nivolumab) that protects activated T cells from exhaustion in peripheral tissues was approved for treatment of patients with melanoma in Japan and in the United States in 2014. In addition, other mAbs against CTLA-4 (tremelimumab), PD-1 (pembrolizumab), and programmed death-ligand 1 (PD-L1, a ligand for PD-1) are currently undergoing clinical trials to evaluate their antitumor efficacy. However, despite clear survival benefits in a subset of tumor patients, other groups of patients are refractory to these single-agent therapies.

Combination therapies comprising immune checkpoint modulators that have different points of action, targeting, for example, the activation and expansion of T cells in lymphoid tissues and the exhaustion and deletion of T cells in the effector site, represent promising strategies for tumor immunotherapy (1). Synergistic antitumor effects in advanced melanoma have been reported with a combination of anti–CTLA-4 and anti–PD-1 mAbs (3). The antitumor efficacy of other combinations of regulators of lymphocyte activation and expansion (e.g., Lymphocyte activation gene-3/LAG-3, OX40/CD134) and of lymphocyte exhaustion and deletion (e.g., T-cell immunoglobulin mucin-3/TIM-3, 4-1BB/CD137, B- and T-lymphocyte attenuator/BTLA, glucocorticoid-induced TNF-receptor/GITR) is currently under investigation. Because immune checkpoint modulators play both positive and negative roles in the immune inhibitory pathway with some redundancy, identification of optimal therapeutic combinations remains a considerable challenge.

Another approach to immune checkpoint modulation involves depleting immunosuppressive leukocyte populations such as forkhead box P3 (Foxp3)+ CD25+ regulatory T cells (Treg), Th2 cells, T regulatory (Tr) 1/3 cells (4), myeloid-derived suppressor cells (MDSC) and indoleamine-2,3-dioxygenase (IDO)+ plasma-cytoid DCs (pDC; refs. 5–7). Several groups have suggested that depletion of CD4+ cells, including Tregs, Th2 cells, Tr1/3 cells, and a subpopulation of MDSCs and pDCs, results in strong antitumor effects in mouse models due to the enhancement of CTL responses (8–12). These antitumor effects may be associated with the modulation of multiple immune checkpoints caused by...
CD4+ cell depletion. However, the relative advantage of CD4+ cell depletion over other immune checkpoint mAb-based treatments remains unclear. Encouraged by the positive reports surrounding the benefits of anti-CD4 mAb treatment in mice, and by the recent clinical data supporting anti–CTLA-4 and anti–PD-1 mAb therapies, here, we examine whether treatments that combine an anti-CD4 mAb and immune checkpoint modulators produce synergistic antitumor activity.

Thus, in the present study, we used comprehensive immunologic analyses to compare the antitumor effects of an anti–CD4-depleting mAb with those of a variety of mAbs against immune checkpoint molecules, including PD-1, PD-L1, PD-L2, CTLA-4, OX40,LAG-3, TIM-3, BTLA, and GITR, in mouse subcutaneous tumor models. We also investigated the antitumor effects of treatments that combined an anti-CD4 mAb and antibodies against these immune checkpoint molecules. We report that treatment with an anti-CD4 mAb alone induces strong antitumor effects and expansion of tumor-specific CD8+ T cells, and that combination of an anti-CD4 mAb with anti–PD-1 or anti–PD-L1 mAbs results in striking synergy in the suppression of tumor growth.

Materials and Methods

Mouse

Seven-week-old female C57BL/6 and male BALB/c mice were purchased from Japan SLC. Fluorescent ubiquitination-based cell-cycle indicator (Fucci) double transgenic mice were generated by crossbreeding FucciG1-#639 and FucciS/G2/M-#474 animals (obtained from Dr. A. Miyawaki through the RIKEN BRC) as described previously (13). Mice transgenic for the gp100 melanoma antigen–specific Pmel-1-TCR or the ovalbumin-specific OT-I TCR were purchased from The Jackson Laboratory. Each experimental group contained 8 mice except where otherwise specified. All animal experiments were conducted in accordance with institutional guidelines with the approval of the Animal Care and Use Committee of the University of Tokyo.

Cell lines and tumor models

B16F10 and Lewis lung carcinoma (LLC) were obtained from the ATCC. Colon 26 was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University. B16F10 cells expressing the truncated form of human low-affinity nerve growth factor receptor (ΔhNGFR/hCD271) were generated by retroviral transduction and two subsequent rounds of in vivo passaging (Supplementary Fig. S1). B16F10 cells (5 x 105/mouse), LLC cells (5 x 105/mouse), and Colon 26 cells (2 x 105/mouse) were inoculated s.c. into the right flanks of C57BL/6 or BALB/c mice. Tumor diameter was measured twice weekly and used to calculate tumor volume (mm3) = [(major axis; mm) x (minor axis; mm)] x 0.5236.

In vivo antibody treatment

Anti-CD4 (clone GK1.5), anti-CD8 (clone YTS169.4), anti–PD-1 (clone J43), anti–PD-L1 (clone 10F.9G2), anti–PD-L2 (clone TY25), anti–OX40 (clone OX-86), anti–CTLA-4 (clone 9D9), anti–LAG-3 (clone C9B7W), anti–BTLA (clone 6A6), anti–TIM-3 (clone RMT3-23), anti–GITR (clone DTA-1), and anti–CD25 (clone PC-61.5.3) mAbs were purchased from BioXcell. Antibodies were injected i.p. at a dose of 200 μg per mouse. Anti-CD4 mAb (200 μg/mouse) was administered in a single dose or in successive doses on days 5 and 9 after tumor inoculation. Immune checkpoint antibodies (200 μg/mouse) were administered on days 4, 8, 14, and 18 after tumor inoculation. Combination treatments with the anti-CD4 mAb and anti-immune checkpoint antibodies were administered under the same conditions as respective single-agent protocols.

Immunohistologic analysis

Immunofluorescent staining was performed as described previously (14–16) using primary antibodies and the appropriate fluorophore-conjugated secondary Abs as listed in Supplementary Table S1, then photographed using an SP5 confocal microscope (Leica Microsystems).

Flow cytometry

Intravascular leukocytes were stained by i.v. injection of fluorophore-conjugated mAb (3 μg/mouse) against CD45 or CD45R.C2 23 minutes before collecting tissues. Single-cell suspensions were prepared by enzymatic or mechanical dissociation of tissues with or without subsequent density separation, as described previously (17, 18). Flow-Count fluorospheres (Beckman Coulter) were used to determine cell numbers and normalize cell concentrations before antibody staining. Cells were pretreated with Fc Block (anti-mouse CD16/CD32 mAb; clone 2.4G2, BioXcell), then stained with mix of fluorophore-conjugated anti-mouse mAbs as indicated in Supplementary Table S1. Data were acquired on a Gallios flow cytometer (Beckman Coulter) and analyzed using FlowJo software (version 9.7.5; FlowJo, LLC). Nonviable cells were excluded from the analysis based on forward and side scatter profiles and propidium iodide staining.

Quantitative reverse transcription real-time PCR

Total RNA was extracted using a RNeasy Mini kit (Qiagen) and converted to cDNA using ReverTae Ace qPCR RT Master Mix with gDNA Remover (Toyobo) according to the manufacturer’s instructions. Real-time quantitative PCR analysis was performed using THUNDERBIRD Probe qPCR Mix or THUNDERBIRD SYBR qPCR Mix (Toyobo), and an ABI 7500 sequence detector system (Life Technologies). The primers used for the PCR reaction are listed in Supplementary Table S2. The expression levels of each gene were normalized to Rp3 expression level for each sample.

Statistical analysis

Unless otherwise stated, data are presented as mean ± SE. Statistical analyses were performed using GraphPad Prism software (version 6.0c; GraphPad Software). For comparisons between groups in the in vivo study, we used one-way ANOVA with the Dunnett post hoc test. For comparisons between the means of two variables, we used paired Student t tests. Comparisons of survival data between groups were made using the log-rank test after Kaplan–Meier analysis. A P value of <0.05 was considered to be statistically significant.

Results

An optimized anti-CD4 mAb treatment protocol exerts robust antitumor effects

We began by optimizing the protocol for anti-CD4 mAb administration in B16F10, LLC and Colon 26 tumor models. Mice bearing subcutaneous tumors received a single i.p. injection of 200 μg anti-CD4 mAb 2 days before (day −2) or 0, 3, 5, or...
9 days after tumor inoculation. In all three models, administration of anti-CD4 mAb on days 3 and 5 significantly suppressed tumor growth (Supplementary Fig. S2A–S2C). B16F10 tumor growth, but not LLC and Colon 26 tumor growth, was also inhibited by mAb administration on days –2 and 0 (Supplementary Fig. S2A). However, the growth of LLC and Colon 26 tumors was not significantly affected by mAb administration at days –2 and 0 (Supplementary Fig. S2B and S2C). Successive administration of the anti-CD4 mAb on days 5 and 9 resulted in the greatest inhibition of tumor growth in all three models (data not shown).

Doses of anti-CD4 mAb (3.1 or 12.5 μg/mouse) that were insufficient to cause CD4 lymphocyte depletion had no inhibitory effect on tumor growth in the melanoma model (Supplementary Fig. S2D and S2E). On the basis of these results, for subsequent studies, we adopted a protocol of administering the anti-CD4 mAb at a dose of 200 μg/mouse successively on days 5 and 9 after tumor inoculation.

We next compared the antitumor effects of the anti-CD4 mAb against those of a variety of immune checkpoint mAbs (PD-1, PD-L1, PD-L2, CTLA-4, OX40, LAG-3, TIM-3, BTLA, and GITR) in the B16F10 model, because melanoma is a major target of anti-immune checkpoint antibody therapy. We found that twice-weekly injections of immune checkpoint antibodies were sufficient to produce the same level of antitumor effect as achieved with daily injections (data not shown). Among the mAbs tested, the anti-CD4 mAb was the most effective single-agent treatment in terms of tumor growth inhibition and survival (Fig. 1A–C). Collectively, these results confirm the potent antitumor effects of anti-CD4 mAb treatment in mice and reveal a surprising advantage of anti-CD4 mAb treatment over immune checkpoint mAb therapy.

Anti-CD4 mAb treatment depletes CD4⁺ T cells and pDCs

To determine which cells are depleted by anti-CD4 mAb therapy, we next examined changes in cell populations with immunosuppressive potential following anti-CD4 mAb administration at day 5 in mice bearing B16F10 tumors. Flow cytometric analysis revealed that numbers of CD4⁺ T cells, including Foxp3⁺ Tregs, decreased 50- to 100-fold over days 2 to 9 following anti-CD4 mAb administration (7 to 14 days after tumor inoculation), as compared with cell numbers in phase-matched untreated tumor-bearing mice (Supplementary Fig. S3A–S3C). When LLC tumor-bearing mice were administered anti-CD4 mAb on days 5 and 9, CD4⁺ T cells disappeared from the blood until at least day 15 after the first mAb administration (Supplementary Fig. S3D). pDCs, a subset of which are positive for CD4 and have been implicated in the suppression of antitumor immune responses (7), also decreased 3- to 10-fold over days 2 to 9 following mAb treatment (Supplementary Fig. S3A–S3C). MDSC subpopulations, including neutrophils and Ly-6Ghi or Ly-6Cmo monocytes, were not significantly affected by mAb treatment (data not shown). These results indicate that CD4⁺ T cells (including Tregs) and pDCs are the targets of anti-CD4 mAb therapy.

Anti-CD4 mAb treatment increases the number of tumor-infiltrating CD8⁺ T cells

We next investigated the effects of anti-CD4 mAb therapy on tumor-infiltrating CD8⁺ T-cell populations. Intravascular staining (IVS) is a technique that allows circulating leukocytes present in tissue blood vessels (which represent a proportion of total leukocytes recovered) to be distinguished from cells...
actually infiltrating the parenchyma of tissues, including tumors (19). In untreated B16F10 tumors, about 15% of CD8\(^+\) T cells were positive for IVS, and the frequency of PD-1\(^+\)CD137\(^+\) tumor-reactive cells (20) was about 10-fold lower in this population than in the IVS-negative parenchymal cell population (Supplementary Fig. S4A and S4B). Anti-CD4 mAb treatment significantly increased the frequency and number of IVS-CD45\(^+\)CD8\(^+\) T cells in the tumor (Fig. 2A and B). The increased number of CD8\(^+\) T cells in the tumors of anti-CD4 mAb-treated mice was also evident in histologic sections (Fig. 2C). Furthermore, the IVS\(^+\)CD8\(^+\) T cells induced by anti-CD4 mAb treatment contained a higher proportion of PD-1\(^+\)CD137\(^+\) tumor-reactive cells (Fig. 2D and E), had greater potential to produce IFN\(\gamma\) in response to \textit{ex vivo} PMA/ionomycin stimulation (Fig. 2F and G), and showed higher specific killing activity against B16F10 tumor cells (Supplementary Fig. S5A–S5C), compared with T cells from the untreated group. In the LLC and Colon 26 tumor models, anti-CD4 mAb-treated mice displayed decreased tumor growth, systematically increased CD8\(^+\)CD44\(^{hi}\)PD-1\(^-\) T cells, and upregulation of LAG-3, TIM-3, and CTLA-4 in tumor-infiltrating CD8\(^+\) T cells (Supplementary Fig. S6A–S6D). Collectively, these results suggest that anti-CD4 mAb treatment enhances antitumor CD8\(^+\) T-cell responses and induces a shift toward type I immunity within the tumor.

**Figure 2.** Anti-CD4 mAb treatment increases the number of tumor-infiltrating CD8\(^+\) T cells. Mice bearing B16F10 (A, B, D–G) or B16F10-\textit{α}LNNGFR (C) tumors were injected i.p. with anti-CD4 mAb on days 5 and 9, and tumor-infiltrating CD8\(^+\) T cells were analyzed on day 14 after tumor inoculation. Control mice received an injection of vehicle only. For flow cytometric analyses, mice were given an i.v. injection of anti-CD45.2 Ab 3 minutes before the collection of tissues to enable identification of cells in the blood compartment (IVS). A, flow-cytometry plots of parenchymal leukocyte compartments (CD45\(^-\)IVS-CD45.2\(^+\)). B, the number of parenchymal CD8\(^+\) T cells in tumor. C, distribution of CD8\(^+\) T cells in the tumor. Green, CDB; red, \textit{α}LNNGFR; blue, propidium iodide (PI). Enlargements in white boxes show nonnecrotic areas, yellow box shows necrotic area; scale bar, 200 \(\mu\)m. D, flow-cytometry plots and frequencies (E) of PD-1\(^+\)CD137\(^+\) tumor-reactive cell population, compared with the parenchymal CD8\(^+\) T-cell population following \textit{ex vivo} restimulation with PMA and ionomycin. Data represent mean \(\pm\) SE of 4 mice per group and are representative of at least four independent experiments. Numbers in flow-cytometry plots indicate mean frequencies within live cells (A) or parental populations (D and F). ***, \(P < 0.001\) (compared with control).

**Anti-CD4 mAb treatment promotes expansion of tumor-specific CD8\(^+\) T cells in the draining lymph node.** To further investigate the effects of anti-CD4 mAb treatment on tumor-specific CD8\(^+\) T-cell responses, we adoptively transferred melanoma antigen-specific Pmel-1 TCR transgenic CD8\(^+\) T cells (21) into mice 1 day before inoculation with B16F10 tumors (day –1; Supplementary Fig. S7A and S7B). On day 14 after tumor inoculation, numbers of Pmel-1 CD8\(^+\) T cells in the blood, draining lymph node (dLN), non-dLN (ndLN), spleen and tumor were 10- to 100-fold higher in anti-CD4 mAb-treated mice compared with that in untreated mice (Supplementary Fig. S7C and S7D). As tumors grown, Pmel-1 CD8\(^+\) T-cell numbers were unchanged or decreased in untreated group mice, whereas Pmel-1 CD8\(^+\) T-cell numbers increased in anti-CD4 mAb-treated mice (Supplementary Fig. S7E). To determine the site of Pmel-1 CD8\(^+\) T-cell expansion, we administered bromodeoxyuridine (BrdUrd) 1 hour before collecting tissues. The number of BrdU\(^+\)-polarizing Pmel-1 CD8\(^+\) T cells in the dLN far outnumbered those in the tumor, irrespective of anti-CD4 mAb treatment (Supplementary Fig. S7F and S7G). Importantly, proliferating cell numbers decreased between days 9 and 14 in untreated mice, but increased in anti-CD4 mAb-treated mice (Supplementary Fig. S7H). Similar CD4 depletion–induced proliferation was also observed in endogenous polyclonal CD8\(^+\) T cells (data not shown). These data suggest that anti-CD4 mAb treatment protects tumor-reactive
CD8⁺ T cells from deletion, a mechanism of peripheral tolerance in which the continuous and excessive exposure of antigen-specific T cells to cognate antigens eventually results in the loss of the antigen-specific T-cell clones.

To confirm the effects of anti-CD4 mAb treatment on the proliferation of CD8⁺ T cells, we used fluorescent ubiquitination-based cell-cycle indicator (Fucci) double transgenic mice. In Fucci mice, Fucci-orange (mKO2) and Fucci-green (mAG) are expressed reciprocally in the G0–G1 and S–G2–M phases of the cell cycle, respectively (13, 18). In the B16F10 tumor model, anti-CD4 mAb treatment significantly increased the proportion of mAG⁺ proliferating cells among CD8⁺ CD44hi T cells in both the dLN and non-dLN, compared with the proportion of these cells in untreated control mice (Supplementary Fig. S7I and S7J).

To determine whether this CD4 depletion–induced proliferation was specific for tumor-specific CD8⁺ T cells or was a tumor antigen-independent response such as homeostatic proliferation (22), we adoptively transferred a CFSE-labeled mixture of Pmel-1, ovalbumin-specific OT-I, and polyclonal CD8⁺ T cells into B16 tumor-bearing or tumor-free mice with or without anti-CD4 mAb treatment (Supplementary Fig. S8A). Pmel-1, but not OT-I or polyclonal CD68⁺ T cells, selectively proliferated in the dLN of B16 tumor-bearing mice (Supplementary Fig. S8B–S8E). These results indicate that CD4 depletion–induced T-cell expansion is specific for tumor-specific CD8⁺ T cells. Collectively, these results suggest that anti-CD4 mAb treatment systemically increases the availability of tumor-specific CD8⁺ T cells by enhancing their proliferation in the dLN in a tumor-associated antigen-dependent manner.

Enhanced CD8⁺ T-cell responses underlie the antitumor effects of anti-CD4 mAb treatment

To determine whether enhanced CTL responses are responsible for the antitumor effects of anti-CD4 mAb treatment, we administered the anti-CD4 mAb together with an anti–CD8-depleting mAb. When the anti–CD8-depleting mAb was administered together with the anti-CD4 mAb, the inhibitory effect of anti-CD4 mAb treatment on tumor growth was completely reversed (Fig. 3A and B). We also investigated whether treatment with an anti–CD25-depleting mAb, which is widely used to deplete Foxp3⁺ CD25⁺ Tregs in mice (23), could produce the same effect as anti-CD4 mAb treatment. Under our administration protocol, tumor growth in the anti-CD25 mAb-treated group was almost equivalent to that observed in untreated mice (Fig. 3A and B). These results suggest that the tumor-specific CD8⁺ T cells that are induced by anti-CD4 mAb treatment are responsible for the antitumor effects of the treatment, and that anti-CD4 mAb treatment might deplete immunosuppressive populations more efficiently than anti-CD25 mAb treatment.

Combination treatment with anti-CD4 and anti–PD-1 or anti–PD-L1 mAbs synergistically enhances antitumor effects

Next, we examined whether synergistic antitumor effects could be achieved by supplementing anti-CD4 mAb treatment with various immune checkpoint mAbs, particularly those targeting the exhaustion and deletion phase of the immune response. We devised a combination treatment protocol of anti-CD4 mAb with immune checkpoint antibodies as depicted in Fig. 4A. Strikingly, combination treatment with anti-CD4 and anti–PD-L1 mAbs, and to a lesser extent anti-CD4 and anti–PD-1 mAbs, resulted in dramatic synergistic inhibition of tumor growth in the B16F10 melanoma model (Fig. 4B and C). Combination treatment with anti-CD4 and anti–CTLA-4, anti–TIM-3, anti–BTLA, and anti-GITR mAbs also had additive or synergistic effects (Fig. 4B and C), but anti–PD-L2, anti–OX40 and anti–LAG-3 mAbs produced no synergistic antitumor effect when combined with the anti-CD4 mAb (Fig. 4B and C). Survival was also prolonged by combination treatment with anti-CD4 and anti–PD-L1 mAbs compared with anti-CD4 mAb monotherapy, but not by other combinations of anti-CD4 and immune checkpoint mAbs (Fig. 4D). Importantly, depletion of CD8⁺ T cells completely abrogated the tumor growth inhibition induced by the combination of anti-CD4 and anti–PD-1 or anti–PD-L1 mAbs, indicating that CD8⁺ T cells play a critical role in the antitumor effects of the combination treatment (Fig. 4E).

To determine whether the synergistic antitumor effects of anti-CD4 and anti–PD-1 or anti–PD-L1 mAb treatment are common to other tumor types and mouse strains, we examined the effect of combination treatment in the Colon 26 subcutaneous tumor model in BALB/c mice. The anti–PD-1 or anti–PD-L1 mAb treatment alone did not inhibit tumor growth, whereas combination treatment with anti-CD4 and anti–PD-1 or anti–PD-L1 mAbs resulted in strong synergistic inhibition of tumor growth (Fig. 5A and B). These effects were completely reversed by treatment with an anti–CD8-depleting mAb (Fig. 5B). Notably, we observed complete remission in 3 of 10 mice treated with the anti-CD4/anti–PD-1 mAb combination, and in 6 of 10 mice treated with the anti-CD4/anti–PD-L1 mAb combination. In addition, the 6 mice that rejected the tumor in the anti-CD4/anti–PD-L1 mAb-treated group were resistant to rechallenge with Colon 26 tumor cells at a dose five times higher than that used in the initial inoculation (Fig. 5C). Collectively, these results indicate that combination
Combination treatment with anti-CD4 and anti–PD-1 or anti–PD-L1 mAbs has synergistic antitumor effects. Mice bearing B16F10 tumors received anti-CD4 mAb, anti-immune checkpoint mAb, or a combination of these, according to the treatment schedule shown in A. B, tumor volume on day 16 (left) or 15 (right); \( \alpha \text{C3}, P < 0.05; \alpha \text{C3/C3}, P < 0.01; \alpha \text{C3/C3/C3}, P < 0.001 \) (compared with control); \( \# , P = 0.021 \) (compared with \( \alpha \text{CD4} \)). C, tumor growth curves. D, survival plots representative of two independent experiments; \( * , P < 0.05; ** , P < 0.01; *** , P < 0.001 \) (comparisons as indicated). E, anti-CD8 mAb was administered together with anti-CD4 mAb and tumor volumes were measured on day 16; \( \dagger , P < 0.05 \) (compared with control). Data, mean ± SE of 8 mice per group.
treatment with anti-CD4 and anti–PD-1 or anti–PD-L1 mAbs has a dramatic and robust antitumor effect that is mediated by antitumor CD8⁺ T cells.

Blockade of the PD-1/PD-L1 signaling axis increases the number of PD-1⁺ tumor-reactive CD8⁺ T cells in the circulation.

Finally, we investigated the cellular and molecular mechanisms underlying the synergy between anti-CD4 and anti–PD-1 or anti–PD-L1 mAbs in the B16F10 melanoma model. Quantitative RT-PCR analysis of whole tumor tissue demonstrated that anti-CD4 mAb treatment alone augmented expression of the antitumor cytokine genes Ifng and Tnf, the IFNγ-inducible genes Cxcl10 and Cd274/PD-L1 (24, 25), and genes encoding the proapoptotic molecules Fasl, Ptf1/perforin, and GzmB/Granzyme B, compared with the expression levels of these genes in untreated tumors (Supplementary Fig. S9A and S9B). The upregulation of PD-L1 by anti-CD4 mAb treatment was also observed at the protein level (Supplementary Fig. S9C). However, no additive or synergistic effects on gene expression were observed in groups receiving combination treatment with anti-CD4 and anti–PD-1 or PD-L1 mAbs. Consistent with these results, the proportion of IFNγ-producing and TNFα-producing cells within the tumor-infiltrating CD8⁺ T-cell population was equivalent between mice receiving anti-CD4 mAb alone and mice receiving the combination of anti-CD4 and anti–PD-1 or anti–PD-L1 mAbs (data not shown).

We next analyzed the effects of anti–PD-1 and anti–PD-L1 mAbs on the PD-1⁺ CD8⁺ T cells that increased in number in the systemic circulation in response to anti-CD4 mAb treatment. We examined cell populations expressing the effector/memory T-cell marker CD44 and the activation marker CD137. Combination treatment with anti-CD4 and anti–PD-L1 mAbs increased the frequency of CD44⁺PD-1⁺ cells among CD8⁺ T cells in the blood, dLN and non-dLN, compared with that in mice receiving the anti-CD4 mAb alone (blood data shown in Fig. 6A and B). In blood CD8⁺ T cells, expression levels of PD-1 on cells within the CD44hi PD-1⁺ population and the frequency of PD-1⁺ CD137⁺ cells were significantly higher in mice that received the combination of anti-CD4 and anti–PD-L1 mAbs compared with the corresponding expression levels and frequency in mice that received the anti-CD4 mAb alone (Fig. 6A–C). In contrast, combination treatment with anti-CD4 and anti–PD-1 mAbs decreased the frequency of the CD44hiPD-1⁺ population among blood CD8⁺ T cells, and decreased the expression levels of PD-1 on cells within the CD44hi PD-1⁺ population (Fig. 6A, E, and F). However, the frequency of the CD44hiCD137⁺ tumor-reactive cell population was higher in mice receiving the combination of anti-CD4 and anti–PD-1 mAbs compared with mice receiving the anti-CD4 mAb alone (Fig. 6A, E, and F), suggesting that anti–PD-1 mAb treatment does not actually decrease the number of tumor-reactive CD8⁺ T cells in the blood, but rather decreases the level of PD-1 expression on these cells. On the other hand, the frequency of PD-1⁺ cells among tumor-infiltrating CD8⁺ T cells in anti-CD4 mAb-treated mice was not affected by treatment with anti–PD-1 or anti–PD-L1 mAbs (Fig. 6D and G).

**Discussion**

The recent success of anti–CTLA-4 and anti–PD-1 mAb therapies in the clinic has highlighted the potential of immunotherapy for the treatment of cancer (2, 3, 26–29). However, the development of immunotherapy for widespread clinical use remains in its early stages. Extensive efforts have been directed toward enhancing endogenous antitumor immunity by dampening the influence of immunosuppressive mechanisms. Treatment strategies have included combinations of antibodies with other antibodies and with other immunotherapies or anticancer therapeutics. In the present study, we demonstrate that antibody-mediated depletion of CD4⁺ T cells from tumor-bearing mice results in enhanced polyclonal PD-1⁺ CD137⁺ tumor-reactive and monoclonal tumor-specific Pmel-1 CD8⁺ T-cell responses, and strong inhibition of tumor growth. Combination treatment with the anti-CD4 mAb and various immune checkpoint mAbs, particularly anti–PD-1 and anti–PD-L1 mAbs, revealed striking synergy in suppressing tumor growth and prolonging survival.

Several previous reports have described antitumor activity of anti-CD4 mAb treatment in solid tumor models in C57BL/6 mice, including subcutaneous tumors induced by inoculation with B16 melanoma cells (9, 11, 12), recurrent TC1 lung cancer cells (30), or embryo cells expressing the adenovirus-derived E1A protein (10). Although the efficacy of immunotherapy in mouse tumors...
models often depends on tumor type, taken together, these reports from independent groups and our results from the present study suggest that anti-CD4 mAb treatment is likely to have broad-spectrum antitumor activity against solid tumors. Optimization of the anti-CD4 mAb administration protocol revealed robust antitumor effects when mice received the mAb on days 3 or 5, rather than when mice receive the mAb before tumor inoculation (day –2). These results suggest that pretreatment is not necessary. However, priming and/or the preexistence of activated CD8+ T cells are important for effective anti-CD4 mAb therapy. Although the mechanistic link between the timing of anti-CD4 antibody administration and the efficacy of treatment remains to be elucidated, administration of the antibody to patients with early-stage cancer or whose tumor burden has been reduced by surgical resection, irradiation or chemotherapeutics is likely to be most beneficial.

A dose of anti-CD4 mAb sufficient to deplete most CD4+ cells was required in order for antitumor effects to be observed. The CD4+ cell population includes Foxp3+CD25+ Tregs, Th2 cells, Tr1/3 cells (4), and IDO+ immunosuppressive pDCs (7). Considering that markedly increased proliferation of tumor-specific CD8+ T cells was observed in the dLN, anti-CD4 mAb treatment is likely to augment proliferation of tumor-reactive CD8+ T cells through the removal of these CD4+ immunosuppressive cells from the dLN. In addition, anti-CD4 mAb treatment increased the proportion of PD-1+CD137+ tumor-reactive cells and IFNγ-producing cells among tumor-infiltrating CD8+ T cells in the B16F10 model, suggesting that anti-CD4 mAb treatment augmented both the quantity and quality of tumor-specific CD8+ T-cell responses. We recently demonstrated that IFNγ- and TNFα-induced cell-cycle arrest is an important mechanism underlying the antitumor effects induced by tumor-specific CD8+ T-cell transfer (31). The shift toward IFNγ-dominant type 1 immunity, which was evident in the strong induction of IFNγ and TNFα in tumor-infiltrating CD8+ T cells after anti-CD4 mAb treatment, is likely to play a central role in the antitumor effects that we observed (32). Notably, depletion of CD25+ Tregs by administration of an anti-CD25 mAb on days 5 and 9 after tumor inoculation did not reproduce the antitumor effect of anti-CD4 mAb treatment. Because some Foxp3+ Tregs have low-to-negative CD25 expression, residual Foxp3+CD25−/lo Tregs may have contributed to this discrepancy. Moreover, the antitumor effects of anti-CD25 mAb treatment have been reported to be optimal when the mAb is administered before tumor inoculation (33, 34), because when it is administered after tumor inoculation, the anti-CD25 mAb depletes not only Tregs but also other activated lymphocytes expressing CD25. The involvement of Treg and other CD4+ immunosuppressive populations in the suppression of CD8+ T-cell–mediated antitumor responses remains to be elucidated.

The synergy that occurs in combination treatment with anti-CD4 and anti–PD-1 or anti–PD-L1 mAbs is likely due to the blockade of PD-1/PD-L1 signaling in PD-1+ activated CD8+ T cells that are induced by anti-CD4 mAb treatment. We did not detect any synergistic effect in terms of the quantity and quality of the tumor-infiltrating CD8+ T-cell response promoted by anti-CD4 and anti–PD-1 or anti–PD-L1 mAb treatment. However, the frequency of the PD-1+CD137+ and CD44hiCD137+ tumor-reactive populations increased among CD8+ T cells in the blood upon blockade of the PD-1/PD-L1 signaling axis. Considering...
that T cells continuously traffic between peripheral and secondary lymphoid tissues via the lymph–blood circulation, the blockade of PD-1/PD-L1 signaling may prevent exhaustion or deletion of tumor-reactive PD-1+CD8+ T cells in the tumor and allow them to migrate into the dLN, thus sustaining antitumor CD8+ T-cell responses. In addition, anti-CD4 mAb treatment increased the number of IFNγ-producing PD-1+CD8+ T cells in the tumor, resulting in the upregulation of IFNγ-inducible genes, including PD-L1. Although the shift toward IFNγ-dominant type-1 immunity within the tumor contributes to the inhibition of tumor growth, it also promotes the exhaustion or deletion of tumor-infiltrating PD-1+CD8+ T cells by enhancing PD-1/PD-L1 signaling. It is therefore likely that the synergy of the anti-CD4 and anti–PD-1 or anti–PD-L1 mAb combination treatment arises due to the blockade of this adverse negative feedback mechanism.

We are in the process of developing a humanized anti-CD4 mAb with potent antibody-dependent cell-mediated cytotoxicity as an anticancer therapeutic. Because CD4+ T cells play important roles in both humoral and cellular immunity, the heightened risk of infectious diseases that may be associated with transient CD4+ T-cell depletion should be carefully evaluated in clinical trials. In addition, trials should seek to maximize clinical efficacy and safety through rigorous optimization of the antibody administration protocol. In preclinical studies in nonhuman primates, no serious adverse effects were detected after several weeks of treatment with our humanized anti-human CD4 mAb that resulted in CD4+ T-cell depletion. In addition, no severe adverse effects have been observed during phase II clinical trials for T-cell malignancy with long-term administration of other humanized anti-CD4 mAbs (35, 36). Preexisting humoral immune mediators, such as immunoglobulin, plasma cells, and memory B cells, CD8+ T-cell responses, and unimpaired natural immunity, are likely to provide basal protection against infectious diseases during CD4+ T-cell-depleting therapies. On the other hand, consideration should also be given to the potential for the acute exacerbation of chronic diseases associated with viral infection (e.g., hepatitis C and B) due to excessive activation of effector and memory CD8+ T cells after CD4+ cell depletion.

In conclusion, our study represents the first report of robust antitumor effects of combination treatment with an anti–CD4-depleting antibody and anti–PD-1 or anti–PD-L1 immune checkpoint antibodies in mice. We have also characterized the immunologic bases for the synergy between these agents. Recent clinical trials suggest that anti–PD-1, anti–PD-L1, or anti–CTLA-4 mAbs, or combinations of these agents, are not effective against all types of solid tumors. Our findings suggest that combination treatment with an anti-CD4 mAb and immune checkpoint mAbs, particularly anti–PD-1 or anti–PD-L1 mAbs, is likely to result in greater clinical efficacy against a broader range of cancers.

Disclosure of Potential Conflicts of Interest
S. Ueha has ownership interest (including patents) in IDAC Theranostics. S. Yokochi is a manager and K. Hachiga is a researcher at IDAC Theranostics. K. Matsushima reports receiving a commercial research grant, has ownership interest (including patents), and is a consultant/advisory board member for IDAC Theranostics. No potential conflicts of interest were disclosed by the other authors.

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References


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