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Synergistic Antitumor Effects of Regulatory T Cell Blockade Combined with Pemetrexed in Murine Malignant Mesothelioma

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CD4+CD25+ regulatory T cells (Tregs) can promote the growth of some tumors, but it is unknown whether this is true for all tumors, including malignant pleural mesothelioma. We have previously shown that the existence of Tregs was associated with poor survival in patients with malignant pleural mesothelioma. In this study, using an intrathoracic murine model of malignant mesothelioma (MM), we provide evidence suggesting that Treg blockade could enhance survival when combined with pemetrexed in established tumor. AC29 murine MM cells were injected into the right pleural cavity of CBA mice for tumor development. Four days after the tumor injection, tumor-bearing mice were then treated with pemetrexed alone, Treg blockade alone, or a combination of pemetrexed and Treg blockade. We observed a synergistic antitumor effect of Treg blockade combined with pemetrexed resulting in prolonged survival. The combination of Treg blockade and pemetrexed was associated with decreased tumor-infiltrating Tregs, increased IL-2 production, dendritic cell maturation, and increased CD3+CD8+IFN-γ+ tumor-infiltrating T cells when compared with mice treated with pemetrexed alone or Treg blockade alone. The survival benefit was abrogated if anti-CD8 mAb was administered simultaneously. Likewise, the survival benefit resulting from the combined Treg blockade with pemetrexed was not observed when immunodeficient mice were used. Therefore, this study suggests that Treg blockade combined with pemetrexed can suppress mesothelioma growth in established tumor in vivo through an immune-mediated process. This study also validates a new intrathoracic tumor model of pleural effusion to explore the role of antitumor immunity in murine MM. The Journal of Immunology, 2010, 185: 956–966.

Cytotoxic chemotherapy has been considered to be detrimental to host antitumor immunity because the dose-limiting toxicity of many cytotoxic drugs is myelosuppression represented by neutropenia and lymphopenia. Recent work, however, suggests that chemotherapy may paradoxically augment antitumor immunity and synergistic activity when used in conjunction with immunotherapy (13). The potential beneficial effects of chemotherapy that have been derived from findings in preclinical studies are: 1) increased Ag presentation and activation of dendritic cells (13); 2) promotion of memory cell generation (14); 3) prevention of tolerance induction by tumor cells with increased tumor-specific T cell trafficking (15); and 4) sensitization of tumor cells to cytotoxic T cell lysis (16). Another advantage of combining chemotherapy and immune-based therapy is that they act through distinct mechanisms, thus the combination may avoid tumor cross-resistance to treatment, and each has its own unique toxicity profile.

However, the effect of chemotherapy on Tregs remains largely unknown. Recently, it was reported that cyclophosphamide depletes Tregs, thus decreasing tumor tolerance and eradicating established tumors (17), but the effect of other chemotherapeutic agents on tumor immunity has yet to be clarified. In particular, pemetrexed, when combined with cisplatin, had a significant impact on survival in a randomized clinical trial for MPM (18), but has never been investigated for its effect on Treg. In the current study, our objectives were to determine: 1) the effects of pemetrexed on host tumor immunity; 2) the effects of Treg blockade when combined with pemetrexed; and 3) if there is any synergistic therapeutic effect of Treg blockade and pemetrexed on tumor eradication.

In the current study, we demonstrated that the combination of pemetrexed with Treg depletion could lead to the eradication of
established intrathoracic mesothelioma in mice, whereas pemetrexed and Treg depletion alone had no significant impact on survival. The synergistic effect of pemetrexed and Treg depletion was associated with increased IL-2 production, intratumoral infiltration of CD3+CD8+IFN-γ T cells, and suppression of IL-10 production at the tumor site. The beneficial effect of pemetrexed associated with Treg depletion was abrogated if CD8+ T cells were depleted or immunedeficient mice were used, suggesting that the synergistic effect of pemetrexed and Treg depletion was mediated through the immune system and effector CD3+CD8+ T cells.

Materials and Methods

Mice and tumor cell line

Female CBA mice and female NOD/SCID mice (NOD.CB17-Pkd1<sup>−/−</sup>) at 6–8 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained in a facility at the University Health Network, University of Toronto (Toronto, Ontario, Canada). Animal care and experiments were performed in accordance with institutional and Canadian Institute of Health guidelines. AC29 and AB12, murine mesothelioma cell lines, were kindly provided by Dr. Jay Kolls (University of Pittsburgh, Pittsburgh, PA). They were developed in asbestos-exposed CBA or BALB/c mice by Davis and coworkers (19). Tumor cells were cultured in RPMI 1640 medium containing 10% of FCS, L-glutamate (Invitrogen, Carlsbad, CA), and penicillin-streptomycin. Tumor cells were maintained at 37˚C in a humidified 5% CO2 atmosphere.

Intrathoracic tumor model of malignant mesothelioma

All animal experiments were conducted according to the guidelines of the Committee for Animals of the University Health Network, University of Toronto, and were approved by the Animal Research Ethics Board at the Toronto General Research Institute, University of Toronto. We have developed a relevant murine tumor model of MPM to investigate the tumor immunity locally at the tumor site and to explore the feasibility of Treg blockade as therapeutic immunomodulation. For intrapleural injection, mice were anesthetized by an i.p. injection of 150 mg/kg ketamine and 10 mg/kg xylazine. The skin overlying the right lateral chest wall was shaved and disinfected, and a 5-mm–long skin incision was made on the right anterolateral chest wall. The fascia and muscle were divided, and the pleural cavity through an intercostal space using a 24-gauge Angiocath was aspirated. The skin incision was closed with a 4–0 monofilament suture, and the mice were observed until complete recovery. The surgical procedure was not associated with mortality or morbidity. Our model mimics the human disease in that the tumor develops diffusely within the pleural space and is accompanied by a significant accumulation of pleural fluid 4 d following tumor cell inoculation (Fig. 1A, 1B). Furthermore, the model also demonstrated accumulation of TILs in developed tumors (Fig. 1C) similar to those seen in human mesothelioma tumors in our previous study (12).

All tissue samples (blood, pleural effusion [PE], tumor, spleen, and lung) were collected at day 10 after tumor injection. When mice were sacrificed, blood was obtained from the inferior vena cava followed by sampling of PE. Tumors in the pleural cavity and lungs were collected for analyses. For histology, tumors were fixed with 10% neutral buffered formalin.

Lymphocyte depletion in vivo

Mice underwent i.p. injection with 250 μg anti-CD25 mAb (clone PC61, eBioscience, San Diego, CA) for CD25+ T cell depletion. Normal rat IgG (rat IgG, eBioscience) was used as control. As previously demonstrated by other authors, flow cytometry revealed that CD4+CD25+ T cells were depleted to ∼70–80% of their original counts by 3 d after a single administration of 250 μg PC61 in lymphoid tissues (20). Flow cytometry with PBMCs also revealed that >90% of CD4+CD25+ T cells were depleted for up to 14 d after PC61 injection (250 μg) when compared with PBMCs in IgG-treated mice (Fig. 2A). To deplete CD8+ T cells, mice received 100 μg anti-CD8 mAb i.p. (clone Ly-2, eBioscience). To deplete CD8+ T cells, mice received 100 μg anti-CD8 mAb i.p. (clone Ly-2, eBioscience). FACs tracing of PBMCs and spleen showing adequate depletion of CD3+CD4+ and CD3+CD8+ T cells after 6 d is presented in Fig. 2B and 2C.

Chemotherapy with pemetrexed

Pemetrexed (100 mg/kg) was given i.p. from days 4–8 (5 consecutive d) to tumor-bearing mice to explore the synergistic effect when combined with anti-CD25 Ab or IgG control. The dose and schedule used for pemetrexed in the current study was determined based on previous studies in mice (21, 22).

Flow cytometry

Tumors, PE, PBMCs, and spleens from the tumor-bearing mice were used for flow cytometric analyses. Abs used for flow cytometric analyses were: FITC-, PE-, APC-, or PE-Cy7–labeled anti-mouse CD3e (clone 145-2C11, eBioscience), anti-mouse CD4 (clone L3T4, eBioscience), anti-mouse CD8a (clone 53-6.7, Abcam, Cambridge, MA), anti-mouse CD25 (clone 7D4, Abcam), PE-labeled anti-mouse IFN-γ (clone XMG1.2, eBioscience), APC-labeled anti-mouse CD11c (clone N418, eBioscience), and normal mouse IgG (BD Medical, Sandy, UT). The skin incision was closed with a 4–0 monofilament suture, and the mice were observed until complete recovery. The surgical procedure was not associated with mortality or morbidity. Our model mimics the human disease in that the tumor develops diffusely within the pleural space and is accompanied by a significant accumulation of pleural fluid 4 d following tumor cell inoculation (Fig. 1A, 1B). Furthermore, the model also demonstrated accumulation of TILs in developed tumors (Fig. 1C) similar to those seen in human mesothelioma tumors in our previous study (12).

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Intrathoracic tumor model of malignant mesothelioma

Murine mesothelioma cells (AC29, 0.5 × 10⁶ cells) derived from an asbestos-exposed CBA mouse were inoculated into the right pleural space of CBA mice. A. Tumor development (arrowhead) with massive PE (arrow) mimicking human MM. B. Tumor development on day 7 after intrapleural tumor inoculation (arrowhead). C. TILs in murine model of MM. Immunohistochemical staining for CD3+ lymphocytes (brown). Original magnification ×400.
CD8+ cell depletion. Flow cytometric analysis was performed 6 d after Ab injection. Percentage of CD8+ cells of total CD3+ cells decreased dramatically in both blood and PE (tenfold dilution with PBS) by performing a Ficoll gradient centrifugation according to the manufacturer’s protocol (Ficoll, Cedarlane Laboratories, Burlington, Ontario, Canada). Surgically excised specimens (tumors and spleens) were cut into pieces followed by mechanical disruption using BD Falcon Cell Strainers (70 µm; BD Biosciences, Franklin Lakes, NJ). Subsequently, suspended cells with PBS were processed by Ficoll centrifugation. Cells were stained with multiple Abs panels: anti-CD3, anti-CD4, and anti-CD25; and anti-CD8. A combination of anti-CD11c and anti-CD80 Abs was used to detect matured dendritic cells. Intracellular staining with anti–IFN-γ Ab was used to detect IFN-γ-secreting cells. To determine the proportions of effecter and central memory T cell subsets in the local tumor environment (i.e., tumor and PE), combinations of anti-CD3, anti-CD8, anti-CD44, and anti-CD62L Abs were used. Data were analyzed using EXPO 32 ADC software (Beckman Coulter). Cell sorting to obtain CD3+CD4+CD25+ or CD3+CD62L Abs were used. Data were analyzed using EXPLO 32 ADC software (Beckman Coulter). Cell sorting to obtain CD3+CD4+CD25+ or CD3+CD62L Abs were used. Data were analyzed using EXPO 32 ADC software (Beckman Coulter). Cell sorting to obtain CD3+CD4+CD25+ or CD3+CD62L Abs were used.

CTIL killing assay

 Splenocytes were taken from mice at day 10 after tumor cell injection or mice without tumor cell injection. Subsequently, splenocytes (5.0 × 10⁶ cells/ml) were cultured in the presence of IL-2 (10 ng/ml; eBioscience) in 2 ml complete culture medium for 5 d. On day 3, one half of the medium was changed with complete medium containing IL-2 (10 ng/ml). Viable splenocytes were separated by Ficoll gradient centrifugation. Tumor cells (AC29 or AB12) were then labeled with 100 µCi [³¹C]l for 1 h at 37°C, washed three times, and resuspended in RPMI 1640 medium containing 10% FCS. Splenocytes were incubated with 1.0 × 10⁶ ⁵¹C-labeled target cells/well in triplicate at the indicated E:T ratio (3, 10, 30, and 90, respectively) for 4 h in a V-bottomed 96-well microtiter plate. Spontaneous release was usually <10% of total release. Cytotoxicity was calculated as follows: (Experimental release − Spontaneous release)/(Total release − Spontaneous release) × 100%.

ELISA

Serum and supernatant of PE were collected by centrifugation at 2000 rpm for 10 min and were stored at −80°C until use. All Abs were purchased from eBioscience. ELISA was used to measure protein levels of IL-2, IL-10, TGF-β1, and IFN-γ according to the manufacturer’s protocol. Absorbance values and corresponding cytokine concentrations were determined with an Opsys MR microplate reader (Dynes Technologies, Chantilly, VA) using the Revelation QuickLink software (Thermo Labsystems, Chantilly, VA).

Quantitative RT-PCR

For synthesis of cDNA from total RNA, the GeneAmp RNA PCR Kit (Applied Biosystems, Warrington, U.K.) was used according to the manufacturer’s instruction using a PTC-100 Thermal Cycler (MJ Research, Waltham, MA) programmed at 55°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Gene primers were designed using Primer Express software version 2.0 (Applied Biosystems). Quantitative RT-PCR (QRT-PCR) reactions were performed on a PRISM 9700HT (Applied Biosystems) using 2 µl cDNA (800 ng/µl), 7.5 µl SYBR Green PCR Master Mix (2×) (Applied Biosystems), and 300 nM gene primers in a total volume of 15 µl. Conditions used for QRT-PCR included an initial step of 15 min at 95°C followed by 40 cycles of 15 s at 94°C and 1 min at 60°C. A standard curve of five serial dilutions and a no-template negative control, all in duplicate, were included in each reaction plate. The gene expression levels of target genes were normalized to the level of β2-microglobulin. The gene forward (F) and reverse (R) primers used in the study are: F: 5’-ATA TGT AGT CCT AGC GGC-3’ and R: 5’-GGC TAC GAT GCA GCA AGA GC-3’. The gene expression was analyzed using Ultrasoft software (Gibco, Invitrogen, Carlsbad, CA). The β2-microglobulin was used as an internal control. Graphing and data analysis were performed with JMP 5.0 software (SAS Institute, Cary, NC). Two-sided p values <0.05 were considered to be statistically significant.

Histology and immunohistochemistry

Fresh tumor specimens were fixed in 10% neutral-buffered formalin, processed, and embedded into paraffin blocks. Paraffin-embedded tumor samples were processed into tissue array blocks, which consisted of four cores per case with a core diameter of 1.5 mm using a tissue microarray instrument (Tissue Arrayer IC, Beecher Instruments, Sun Prairie, WI). Tissue array blocks were then cut into 4-µm sections for H&E and immunohistochemical staining. Rabbit anti-mouse CD3ε mAb (DakoCytomation, Glostrup, Denmark) was used to detect TILs. All four spots of each tumor sample were evaluated for TILs, then a minimum of 5 independent areas (up to 10 areas) with the most abundant immunohistochemically positive TILs were selected at a size of 0.0625 mm². Positive cells in the selected areas were counted independently by two investigators (M.A. and Z.Y.) in a blinded fashion. The average numbers of positive TILs were used for statistical analyses.

Survival study

Survival study was carried out in lieu of tumor burden assessment to compare treatment groups. The animals (CBA or NOD.CB17-Prkdcsid mice) were sacrificed when they met predetermined criteria (i.e., tumor growth compromising food and water intake or respiration) established to minimize pain and suffering and were scored as death.

Statistical analyses

Data are presented as means ± SD. Kaplan-Meier survival curves were used to estimate survival rates for different treatment groups and were compared with the log-rank test. One-way ANOVA was used to compare averages of values from multiple groups followed by Dunnett’s test. Comparisons between two groups were performed by the t test. All analyses were performed with JMP 5.0 software (SAS Institute, Cary, NC). Two-sided p values <0.05 were considered to be statistically significant.
Results

Depletion of CD25+ cells before tumor cell injection led to prolonged survival

To determine the effect of CD25+ cell depletion on tumor development, we depleted CD25+ cells before tumor cell injection. Flow cytometric analyses with PE revealed that the proportion of CD3+CD4+CD25+ T cells of total CD3+ T cells (%CD3+CD4+CD25+ T cells) decreased significantly in mice treated with PC61 compared with those treated with rat IgG (Fig. 3A). In contrast, the proportion of CD3+CD8+ T cells of total CD3+ T cells (%CD3+CD8+ T cells) in tumors from mice treated with PC61 was significantly increased compared with those treated with rat IgG (Fig. 3A). mRNA expression of Foxp3 was significantly suppressed in tumors of mice treated with PC61 compared with tumors of mice treated with rat IgG control (Fig. 3B). Foxp3 gene expression was detected in tumor-infiltrating CD3+CD4+CD25+ T cells, but not in tumor-infiltrating CD3+CD8+ T cells (data not shown).

To determine the effect of CD25+ Treg blockade using PC61 on antitumor immune response in tumor-bearing mice, we studied cytokine profiles including both antitumor cytokines (IL-2 and IFN-γ) and immunosuppressive cytokines (IL-10 and TGF-β1). We found that protein levels of IL-2 and IFN-γ in PEs of PC61-treated tumor-bearing mice were significantly higher than in rat IgG-treated or in nontreated mice (Fig. 3C). In contrast, the immunosuppressive cytokine TGF-β1 was significantly suppressed in PEs of PC61-treated tumor-bearing mice when compared with that of nontreated mice (Fig. 3C). We did not see any significant difference in protein levels of IL-10 among groups.

In survival analyses, mice treated with PC61 before tumor cell injection demonstrated significantly longer survival than those treated with rat IgG or with no treatment (Fig. 3D). On the contrary, there was no survival benefit observed when mice were treated with anti-CD4 mAb or anti-CD8 mAb alone (data not shown). Because an increased proportion of CD8+ T cells was observed in tumors of mice treated with PC61 before tumor injection, we then questioned whether the prolonged survival seen in mice treated with PC61 was due to the presence of effector CD8+ T cells. We found that anti-CD8 mAb abrogated the advantageous effect on survival obtained by PC61 (Fig. 3D). These results suggest that CD3+CD8+ effector T cells were necessary for prolonging survival when Tregs were depleted by PC61.

Depletion of CD25+ cells after tumor development did not lead to prolonged survival

The efficacy of PC61 treatment before tumor cell injection on survival and the cytokine profile (i.e., high IFN-γ and low TGF-β1) led us to question whether PC61 treatment after tumor development could lead to prolonged survival. PC61 was therefore administered 4 d after tumor inoculation.

Flow cytometric analyses of PBMCs, tumors, and PEs demonstrated significant decrease of the percentage of CD3+CD4+CD25+ in PEs of PC61-treated tumor-bearing mice when compared with that of nontreated mice (Fig. 3C). We did not see any significant difference in protein levels of IL-10 among groups.

In survival analyses, mice treated with PC61 before tumor cell injection demonstrated significantly longer survival than those treated with rat IgG or with no treatment (Fig. 3D). On the contrary, there was no survival benefit observed when mice were treated with anti-CD4 mAb or anti-CD8 mAb alone (data not shown). Because an increased proportion of CD8+ T cells was observed in tumors of mice treated with PC61 before tumor injection, we then questioned whether the prolonged survival seen in mice treated with PC61 was due to the presence of effector CD8+ T cells. We found that anti-CD8 mAb abrogated the advantageous effect on survival obtained by PC61 (Fig. 3D). These results suggest that CD3+CD8+ effector T cells were necessary for prolonging survival when Tregs were depleted by PC61.
T cells in tumor-bearing mice treated with PC61, but not in mice treated with rat IgG (Fig. 4A). Moreover, a higher percentage of CD3+CD8+ T cells was observed in both tumors and PE in mice treated with PC61 (Fig. 4B). However, PC61 treatment after tumor development failed to demonstrate prolonged survival (Fig. 4C).

PC61 treatment after tumor development failed to induce antitumor cytokines IL-2 and IFN-γ. These cytokines were produced markedly less in mice treated with PC61 after tumor development than in those treated before tumor injection (IL-2: 33.5 ± 8.2 versus 790.5 ± 175.6 pg/ml; p < 0.0001; IFN-γ: 525.6 ± 163.2 versus 1258.9 ± 898.9 pg/ml; p = 0.2). In addition, PC61 treatment after tumor development failed to suppress the production of immunosuppressive cytokine TGF-β1 compared with those treated with PC61 before tumor injection (1234.9 ± 431.7 versus 273.0 ± 100.0 pg/ml; p = 0.02).

Chemotherapy plus CD25+ T cell blockade after tumor development demonstrated a synergistic effect on survival

Pemetrexed combined with CD25+ cell blockade (PC61) was tested to determine if there is any synergistic effect on prolonged survival. Because increased percentage of CD3+CD4+CD25+ T cells were observed after pemetrexed chemotherapy in our murine model (Fig. 5A), we hypothesized that Treg blockade combined with pemetrexed may effectively change the tumor microenvironment and results in better outcome.

In flow cytometric analyses, when PC61 was given with pemetrexed, Tregs were effectively depleted in PBMCs, tumors, and PE (Fig. 5A). The depletion of Tregs was also demonstrated by qRT-PCR in tumors and spleens. Significantly lower mRNA levels of Foxp3 were observed in the group of mice treated with PC61 plus pemetrexed when compared with mice treated with rat IgG plus pemetrexed or no treatment (Fig. 5B). The proportions of CD3+CD8+ T cells were significantly increased in the group of mice treated with pemetrexed plus PC61 in tumors and PEs (Fig. 5C).

FIGURE 4. Depletion of CD25+ cells after tumor cell injection failed to demonstrate antitumor effects. Mice were treated with either PC61 or rat IgG 4 d after tumor cell injection into the right thoracic cavity. Flow cytometric analyses and cytokine measurements were performed at day 10 after tumor cell injection. A. Flow cytometric analysis of percentage of CD3+CD4+CD25+ T cells of total CD3+ T cells in PBMCs (left panel), tumors, and PE (right panel) (n = 6/group). B, Flow cytometric analysis of percentage of CD3+CD8+ T cells of total CD3+ T cells in tumors and PE (n = 6/group). C, Survival analysis. Mice were treated with PC61 alone or rat IgG alone 2 d after tumor cell injection into the thoracic cavity (n = 10/group). *p < 0.05.

Semiquantification of CD3+ TILs by the immunohistochemical staining revealed that the number of tumor-infiltrating T lymphocytes in the group treated with pemetrexed alone was significantly smaller than that in the group treated with pemetrexed plus Treg blockade (Fig. 5D).

These results demonstrate that the combination of pemetrexed plus Treg blockade leads to a dramatic increase in the absolute number of CD8+ T cells within the tumor when compared with the administration of pemetrexed alone (20 ± 7 tumor-infiltrating CD8+ T cells per area versus 5 ± 4 tumor-infiltrating CD8+ T cells per area, respectively; p = 0.001). Furthermore, flow cytometric analyses revealed increased the percentage of CD3+CD8+ IFN-γ+ T cells in the tumors from the group treated with pemetrexed plus Treg blockade compared with that with pemetrexed alone (12.9 ± 1.4% versus 8.3 ± 1.2%; p = 0.02) or PC61 alone (12.9 ± 1.4% versus 9.5 ± 1.5%; p = 0.04; Fig. 5E). Moreover, the estimated CD4+CD25+ tumor-infiltrating T cells per area were significantly smaller in the tumors treated with pemetrexed plus Treg blockade compared with those with no treatment (3.2 ± 0.3 versus 6.3 ± 0.6; p = 0.02). Proportions of naive CD3+CD8+CD44−CD62L+ T cells or central memory CD3+CD8+CD44+CD62L+ T cells did not show statistical difference between the treatment groups including no treatment, PC61 alone, pemetrexed with rat IgG, and pemetrexed plus PC61 (4.4 ± 4.8%, 5.1 ± 4.6%, 5.5 ± 4.1%, 11.1 ± 9.7%; p = 0.6; and 36.9 ± 18.0%, 37.2 ± 26.3%, 39.3 ± 13.4%, 39.0 ± 22.8%, p = 0.9, respectively).

In a survival analysis, we observed significantly better survival in the group of mice treated with PC61 plus pemetrexed compared with those treated with PC61 alone, rat IgG plus pemetrexed, or no treatment (Fig. 6A). The cytokine profiles demonstrated that mice treated with pemetrexed plus Treg depletion by PC61 had significantly higher levels of IL-2 and significantly lower levels of TGF-β1 and IL-10 in the PE when compared with the control group receiving no treatment or to the group receiving pemetrexed only.

FIGURE 5. Depletion of CD25+ T cells following pemetrexed treatment. T cells of total CD3+ T cells were assessed in tumors and PE in mice treated with PC61 alone, pemetrexed plus rat IgG, and pemetrexed plus PC61 (A-C). A, Flow cytometric analysis of percentage of CD3+CD4+CD25+ T cells of total CD3+ T cells in PBMCs (left panel) and tumors and spleens (right panel) (n = 6/group). B, Flow cytometric analysis of percentage of CD3+CD8+ T cells of total CD3+ T cells in tumors and PE (n = 6/group). C, Survival analysis. Mice were treated with PC61 alone or rat IgG alone 2 d after tumor cell injection into the thoracic cavity (n = 10/group). NS = not significant.

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with IgG (Fig. 6B). IFN-γ also tended to be higher in the group receiving pemetrexed and PC61 when compared with the other groups, but did not reach statistical significance.

**Effector CD8**⁺ and CD4⁺ T cells are indispensable to induce the synergistic antitumor effect of pemetrexed plus CD25⁺ T cell blockade

Firstly, we used NOD.CB17-Prkdcscid mice for survival studies with the same treatment protocols to determine whether the synergistic effect of pemetrexed and Treg blockade was mediated by the immune system. In the survival analysis, there was no statistical difference between groups of mice with pemetrexed plus Treg blockade and pemetrexed alone (p = 0.6), suggesting that the immune system is indispensable to induce the synergistic antitumor effect of pemetrexed and Treg blockade (Fig. 7A).

Secondly, to determine whether the beneficial effect of pemetrexed plus Treg depletion was mediated through effector CD8⁺ or CD4⁺ T cells, we selectively depleted CD4⁺ or CD8⁺ T cells from CBA mice. Effector CD8⁺ T cells were shown to be indispensable to eradicate established tumors when pemetrexed is combined with Treg blockade, because the administration of anti-CD8 mAb abolished the favorable effects of Treg blockade combined with pemetrexed on survival (Fig. 7B). The beneficial effect of pemetrexed plus Tregs on survival was also abolished in CD4-depleted mice but to a lesser extent than in CD8-depleted mice (Fig. 7C). The blockade of CD4⁺ combined with PC61 effectively deleted CD4⁺CD25⁺ T cells in the tumors (1.5 ± 1.5%) but also eliminated CD3⁺CD4⁺ T cells (10.1 ± 4.3%). CD3⁺CD8⁺ T cells were completely deleted when anti-CD8 mAb was combined with PC61 (0%). In contrast, the PC61 group demonstrated low percentage of CD4⁺CD25⁺ T cells (1.5 ± 1.5%; p = 0.005) but high CD3⁺CD4⁺ (44.6 ± 3.4%; p < 0.001) and CD3⁺CD8⁺ T cells (29.5 ± 5.4%; p = 0.001), suggesting that the CD25 blockade is the most effective way to eliminate CD4⁺CD25⁺ T cells, but preserve both helper CD4⁺ and cytotoxic CD8⁺ T cells in the tumors. Furthermore, the effect of PC61 was not diminished even if combined with pemetrexed (CD3⁺CD4⁺ T cells: 38.9 ± 17.2%; CD4⁺CD25⁺ T cells: 4.7 ± 1.4%; and CD3⁺CD8⁺ T cells: 27.1 ± 7.0%). In other words, CD4⁺ helper and CD8⁺ cytotoxic T cells were preserved, but CD4⁺CD25⁺ T cells were effectively eliminated with this combined protocol. The administration of pemetrexed alone in CD4-depleted mice or in CD8-depleted mice had no significant impact on survival. Altogether, these results demonstrate that both effector CD8⁺ T cells and effector CD4⁺ T cells were indispensable for host antitumor reaction, and the beneficial effect of pemetrexed plus PC61 on survival was dependent upon the recruitment of CD8⁺ T cells and CD4⁺ T cells within the tumor.
Chemotherapy plus CD25+ T cell blockade facilitates dendritic cell maturation and lymphocyte tumor-specific killing

To determine the effect of combined pemetrexed and Treg blockade on the maturation of dendritic cells in the local tumor environment, we performed flow cytometric analyses using mononuclear cells from the tumors (Fig. 8A). The analyses demonstrated increased proportions of matured CD11c+CD80+ cells in the tumors from mice treated with pemetrexed plus PC61 compared with those with no treatment (68.4 ± 3.0% versus 53.9 ± 12.5%; p = 0.03), or those with PC61 alone (68.4 ± 3.0% versus 58.5 ± 3.6%; p = 0.03). There was also a trend toward higher proportion of matured dendritic cells in the group treated with pemetrexed plus PC61 than in the group treated with pemetrexed alone (68.4 ± 3.0% versus 61.0 ± 6.3%; p = 0.06).

In CTL killing assays, splenocytes from AC29 tumor-bearing mice treated with pemetrexed and PC61 demonstrated significantly higher tumor lyses on AC29 cells when compared with those from mice without treatment (44.3 ± 3.1% versus 15.1 ± 0.4%; p = 0.01; Fig. 8B). The tumor lyses rate of splenocytes from mice with pemetrexed plus PC61 on AC29 cells was also significantly higher than that on AB12 cells (44.3 ± 3.1% versus 27.3 ± 1.1%; p = 0.04).

Discussion

This study demonstrates the synergistic impact of a combination of chemotherapy and immunotherapy on outcome in established intrapleural mesothelioma in mice. We used an innovative strategy by combining pemetrexed with the depletion of Treg using an anti-CD25 mAb (PC61). These results support other recent studies demonstrating the potential effect of a combined approach with chemotherapy and adoptive T cell transfer (25, 26). This is, however, to our knowledge the first time that chemotherapy and depletion of Tregs were shown to act synergistically. The mechanisms leading to the synergistic interaction between chemotherapy and the depletion of Tregs are complex and likely relate to some changes in the tumor microenvironment with increased pro-inflammatory cytokines IL-2 and intratumoral CD3+CD8+IFN-γ+ T cells, enhanced dendritic cell maturation, and a decrease in anti-inflammatory cytokine IL-10 in the group receiving pemetrexed and PC61.

The presence of Tregs is probably one of the major components of the anti-inflammatory microenvironment generated by the tumor to suppress the effector arm of the antitumoral immune response. Tumor cells and surrounding macrophages produce the chemokine CCL22 that mediates trafficking of Treg to the tumor via CCR4 (8, 27). The presence of Tregs and the release of anti-inflammatory cytokines,
such as TGF-β and IL-10, generate an anti-inflammatory milieu that polarizes tumor-associated macrophages and TIL toward M2 macrophage and Th2 T cell phenotypes. M2 and Th2 phenotypes differ from M1 and Th1 phenotypes in terms of function and cytokine release (8). M2 and Th2 release anti-inflammatory cytokines, such as TGF-β and IL-10 cytokines, and promote the anti-inflammatory milieu (28). In contrast, M1 macrophages are activated by IFN-γ and promote a Th1 response with release of proinflammatory cytokines, leading to an antitumoral response. The absence of Tregs may therefore break the tolerance induced by the tumor microenvironment and shift the paradigm from an anti-inflammatory to a proinflammatory response. The proinflammatory tumor microenvironment may then allow cytotoxic CD8+ T cells to reject the tumor.

In our study, the dendritic cell maturation in the local tumor microenvironment was facilitated in the combined pemetrexed and PC61 treatment group compared with those without treatment or PC61 treatment alone. The greater dendritic cell maturation was associated with greater lymphocyte tumor-cell killing on AC29 tumor cells, but not on AB12 (control murine mesothelioma cells). The result indicates that effective AC29 cell-specific tumor recognition and killing were induced at the local tumor site by the combined pemetrexed and PC61 treatment. It is consistent with the fact that more cytotoxic CD8+ T cells were seen in the tumors of mice treated with the combined treatment than in mice treated with chemotherapy or PC61 alone. Hegmans et al. (29) have demonstrated the importance of matured dendritic cells in antitumor immunity in malignant mesothelioma (MM) in which antitumor immune responses were induced by giving tumor lysate-pulsed dendritic cells in patients who underwent cisplatin and pemetrexed-based chemotherapy.

In our model, the proportions of central memory T cell subset were not affected by different treatment regimens. Given that the balance between self-specific memory Tregs and memory effector T cells has appeared to dictate a tolerogenic environment for tumors (30), further phenotypic analyses of memory lymphocyte subsets are required to delineate the effect of Treg blockade on antitumor immunity when combined with chemotherapy.

**FIGURE 7.**  A, Survival analysis using NOD. CB17-Prkdcscid mice. Mice were treated with PC61 alone, pemetrexed plus PC61, or pemetrexed plus rat IgG 4 d after tumor cell injection into the thoracic cavity (n = 8/group). There was no statistical difference between treatment groups. B and C, Impacts of CD4+ or CD8+ T cells on survival of mice treated with Treg blockade (day 4 after tumor inoculation) and pemetrexed chemotherapy (days 4–8). Tumor-bearing mice were treated with pemetrexed plus rat IgG, PC61 plus pemetrexed, PC61 and anti-CD8 mAb plus pemetrexed, or anti-CD8 mAb plus pemetrexed (B) or with PC61 and anti-CD4 mAb plus pemetrexed or anti-CD4 mAb plus pemetrexed (C). The group of mice treated with PC61 plus pemetrexed demonstrated statistically longer survival than other groups.
The combination of chemotherapy with low-dose cyclophosphamide followed by adoptive transfer of tumor reactive lymphocytes led to major improvement in the treatment of refractory metastatic melanoma (26). Low-dose cyclophosphamide is known to inhibit the function of CD4+CD25+Foxp3+ Tregs and may thus have accounted for the augmented immune response observed with this regimen by a change in the tumor microenvironment before injecting tumor reactive lymphocytes (25, 31). The lymphodepleting and immunostimulatory approaches have been tested in murine mesothelioma model by van der Most et al. (32), demonstrating CD8 T cell- and NK cell-dependent antitumor effects after cyclophosphamide chemotherapy. Our results support the immunochemotherapy concept and demonstrate that Treg blockade in combination with chemotherapy can eradicate established tumors by a change in the tumor microenvironment from an anti-inflammatory to a proinflammatory milieu allowing effective antitumoral effect from CD4+ and CD8+ T cells, whereas the administration of chemotherapy alone or Treg blockade alone had no impact on the tumor microenvironment or survival.

Increasing evidence also suggests that the immune system plays a major role in the antitumor effects of conventional chemotherapy (33). The importance of antitumor immune reaction was supported by our experiments using NOD-SCID mice, in which the immuno modulation approach with PC61, with or without pemetrexed, did not show any survival benefit in these immunodeficient mice (Fig. 7A). Hence, better understanding of the interaction between specific chemotherapeutic agents and the immune system can potentially lead to a synergism between both therapies and be associated with a dramatic improvement in tumor response. No study has yet specifically analyzed the effect of pemetrexed on the immune system. However, our results suggest that the beneficial effect of pemetrexed on outcome in mesothelioma could be partially mediated through the activation of effector CD8+ T cells because the depletion of CD8+ T cells in mice receiving pemetrexed alone tended to be associated with a reduction in survival (Fig. 7B).

Anti-CD25 mAb (PC61) was used to block Tregs in our model. Although PC61 is not specifically directed against Tregs, most authors have used this Ab to deplete Tregs in animal models due to the lack of a more specific alternative. Rudge and coworkers (34) reported that CD4+CD25+Foxp3+ Tregs were successfully depleted with 0.25 mg PC61 i.p. and led to tumor rejection with augmented IFN-γ-secreting T cells in their murine mesothelioma model. Blocking Foxp3 with small interfering RNA strands or with antisense oligonucleotides has been used to block Tregs, but these methods have been mostly used for in vitro experiments because of the complexity to deliver them in vivo. In our study, anti-CD25 mAbs do block CD4+CD25+Foxp3+ Tregs because Foxp3 expression was considerably decreased in tumors and spleens of mice treated with PC61. However, the impact of the lack of specificity in Treg depletion is difficult to evaluate. We believe that the potential depletion of activated CD4+ or CD8+ T cells by PC61 played a limited role in our model because the administration of PC61 alone in mice with established tumor had no impact on outcome. The overall mechanism leading to eradication of established mesothelioma by the combination of pemetrexed and PC61 is complex and seems to results from an imbalance among Tregs, the cytokine milieu, and the recruitment of effector antitumoral CD4+ and CD8+ T cells within the tumor.

Secretion of TGF-β1 was suppressed in mice treated with Treg blockade and chemotherapy when compared with those without treatment or with pemetrexed alone. However, it was also suppressed in the group treated with PC61 alone (Fig. 6B), suggesting the suppression of TGF-β1 could be induced without the combined approach. This cytokine suppression may be a key factor for effective antitumor immune reaction in mesothelioma and other tumors (35–38). When Treg blockade after tumor development failed to suppress TGF-β1 production, survival was not prolonged. In addition, we observed that pemetrexed chemotherapy alone could facilitate its production (Fig. 6B). These findings are consistent with previous studies showing that TGF-β production can be induced by antitumor therapies, such as chemotherapy or radiotherapy, and result
in accelerated tumor progression (36, 38). Altogether, the production of TGF-β1 at the local tumor site facilitated by pemetrexed chemotherapy could be suppressed by Treg blockade.

There are some limitations in our study. First, the amounts of immunogenicity differ with the strains of mice, thus our findings using CBA mice with AC29 should be carefully interpreted. Secondly, immune response to pemetrexed could be different between mice and human; mice generally have high thymidine levels and require high doses of pemetrexed because the effect of antifolate antimetabolite, such as pemetrexed, is dependant on the thymidine microenvironment of patients with pancreas or breast adenocarcinoma. The authors have no financial conflicts of interest.

In summary, we have demonstrated an effective antimimmune response by Treg blockade in combination with pemetrexed. The immunomodulation approach with anti-CD25 mAb boosts treatment efficacy by the production of substantial levels of IL-2, facilitated dendritic cell maturation, decreased anti-inflammatory cytokine IL-10 production, and increased tumor-infiltrating CD8+ IFN-γ+ effector T cells. Because the effects of pemetrexed on the host antitumor immune system are still largely unknown, the mechanisms by which the combination of pemetrexed and Treg blockade work will require further studies. In addition, detailed phenotypic characterizations of Tregs (i.e., TGF-β or IL-10 secreting) and tumor-specific CD8+ cytotoxic T cells will be required to elucidate the complex interaction among Tregs, the cytokine milieu, and the recruitment of effector antitumoral T cells.

Disclosures
The authors have no financial conflicts of interest.

References
