Preclinical Development

CTLA-4 Blockade Expands Infiltrating T Cells and Inhibits Cancer Cell Repopulation during the Intervals of Chemotherapy in Murine Mesothelioma

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Abstract

Cancer immunotherapy has shown promising results when combined with chemotherapy. Blocking CTLA-4 signaling by monoclonal antibody between cycles of chemotherapy may inhibit cancer cell repopulation and enhance the antitumoral immune reaction, thus improve the efficacy of chemotherapy in mesothelioma. The impact of CTLA-4 blockade on the early stage of tumor development was evaluated in a subcutaneous murine mesothelioma model. CTLA-4 blocking antibody was administered following each cycle of chemotherapy, and monotherapy was included as controls. Antitumor effect was evaluated by tumor growth delay and survival of the animals. Tumor cell repopulation was quantified by bromodeoxyuridine incorporation and Ki67 by immunohistochemistry and/or flow cytometry. In vitro cell killing was determined by classic chromium-released assay, and reverse transcription PCR (RT-PCR) was carried out to determine the gene expression of associated cytokines. Anti-CTLA-4 monoclonal antibody was able to inhibit tumor growth at early stage of tumor development. Antitumor effect was achieved by administration of CTLA-4 blockade between cycles of chemotherapy. Tumor cell repopulation during the intervals of cisplatin was inhibited by CTLA-4 blockade. Anti-CTLA-4 therapy gave rise to an increased number of CD4 and CD8 T cells infiltrating the tumor. RT-PCR showed that the gene expression of interleukin IL-2, IFN-γ, granzyme B, and perforin increased in the tumor milieu. Blockade of CTLA-4 signaling showed effective anticancer effect, correlating with inhibiting cancer cell repopulation between cycles of chemotherapy and upregulating tumor-infiltrating T lymphocytes, cytokines, and cytolytic enzymes in a murine mesothelioma model.

Mol Cancer Ther; 11(8); 1809–19. ©2012 AACR.

Introduction

Immunosuppressive components such as regulatory T cells (Treg), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), myeloid-derived suppressor cell (MDSC), and to some extent, tumor-infiltrating macrophages, play critical roles in tumor tolerance through a variety of mechanisms (1–5). Therefore, inhibitory checkpoints of immune regulation provide potential targets for cancer immunotherapy. CTLA-4, also known as CD152, is a member of the CD28/B7 immunoglobulin superfamily of immune regulatory molecules. It shares its 2 ligands (B7-1 and B7-2) with its costimulatory counterpart CD80 and CD86, and to some extent, tumor-infiltrating macrophages, play critical roles in tumor tolerance through a variety of mechanisms (1–5). Therefore, inhibitory checkpoints of immune regulation provide potential targets for cancer immunotherapy. CTLA-4, also known as CD152, is a member of the CD28/B7 immunoglobulin superfamily of immune regulatory molecules. It shares its 2 ligands (B7-1 and B7-2) with its costimulatory counterpart CD28 (4, 6). CTLA-4 and CD28 and their ligands B7-1 (CD80) and B7-2 (CD86) are critically important for the initial activation of naive T cells and regulation of the clonal composition of the responding repertoire following migration of activated dendritic cells to lymphoid organs (7–9). Taken together, these 4 molecules are perhaps the most important cofactors functioning in an immunologic cascade, providing signals that are crucial in T-cell activation and tolerance. Change in T-cell activation by blocking negative signaling receptors such as CTLA-4 is one approach to overcoming tumor-induced immune tolerance. Therefore, the discovery of CTLA-4 as a key negative regulator in immune activity enabled to develop novel therapy to target this signaling molecule (10, 11). Anti-human CTLA-4 antibodies ipilimumab and tremelimumab prolong antitumor immune responses and lead to durable antitumor effects (12–14).

Current understanding of CTLA-4 function in T-cell responses in vitro and in preclinical murine models made it possible to initiate application of CTLA-4 blockade as a novel immunotherapy for cancer (15, 16). Other preclinical studies also show that CTLA-4 blockade can act synergistically with other treatment modalities such as irradiation, cryotherapy, and chemotherapy (15–17). The
initial clinical experience with antibody to CTLA-4 in human trials has been reported along with a perspective on adverse events observed with CTLA-4 blockade in some cancers (18–20). One neglected area in cancer research that has recently been highlighted is the importance of repopulation of surviving cancer cells between courses of chemotherapy and radiotherapy (21, 22). The rate of surviving cell repopulation may increase during fractionated radiotherapy and between cycles of chemotherapy, limiting the ability to control tumors (23). Repopulation is likely to be more important with chemotherapy than with radiation therapy because of the longer intervals between cycles of treatment and is a potential cause of clinical failure to chemotherapy. Accelerating repopulation after sequential courses of chemotherapy could lead to regrowth of tumors after initial shrinkage without any change in the intrinsic sensitivity of the cells to the drugs used (23). Thus, accelerating repopulation may be an important cause of clinical drug resistance. Therefore, inhibition of this process may improve the efficacy of chemotherapy (24, 25).

Current evidence has shown that CTLA-4 blockade has synergistic effect with chemotherapy in some animal studies and clinical trials (11–14, 21). However, the optimal timing of such therapies has not been confirmed, especially in mesothelioma. To our best knowledge, the impact of CTLA-4 blockade-based immunotherapy on cancer cell repopulation between cycles of chemotherapy has not been reported. In this study, we administered CTLA-4 blocking monoclonal antibody between cycles of chemotherapy. Accelerating repopulation may increase during fractionated radiotherapy, surviving cancer cells between courses of chemotherapy and between cycles of chemotherapy, limiting the ability to control tumors (23). Repopulation is likely to be more important with chemotherapy than with radiation therapy because of the longer intervals between cycles of treatment and is a potential cause of clinical failure to chemotherapy. Accelerating repopulation after sequential courses of chemotherapy could lead to regrowth of tumors after initial shrinkage without any change in the intrinsic sensitivity of the cells to the drugs used (23). Thus, accelerating repopulation may be an important cause of clinical drug resistance. Therefore, inhibition of this process may improve the efficacy of chemotherapy (24, 25).

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Materials and Methods

Murine mesothelioma cells and animal model

Murine malignant mesothelioma cell lines AB12 and AC29, derived from an asbestos-induced tumor in a BALB/c and CBA/J mouse, respectively, were kindly provided by Dr. Jay Kolls, University of Pittsburgh, Pittsburgh, PA, in 2008 (2, 26). Both cell lines were revived from the original stocks and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin and streptomycin. No further authentication was done. The cultures were maintained at 37°C in an atmosphere containing 5% CO2. AB12 and AC29 cells (2 × 105) were injected subcutaneously into the right flank of female BALB/c and CBA/J mice, which were provided by The Jackson Laboratory. All procedures followed the animal care regulations of University Health Network after approval by the Research Ethic Board.

Treatment at the early stage of tumor development with anti-CTLA-4 monoclonal antibody

Mice were treated with anti-mouse CTLA-4 monoclonal antibody (clone: 9H10; eBioscience) 100 μg per dose by i.p. injection on days 1 and 3 after tumor cell injection (i.e., before the development of a palpable tumor). The impact on tumor growth was observed twice weekly. T-cell population and function in tumor and spleen were quantitatively evaluated by flow cytometry.

In vitro generation of tumor-specific CTLs by chromium-release assay

Splenocytes derived from naive or AB12-bearing mice at 7 days after tumor challenge were pooled into 24-well plates at a concentration of 2.5 × 106 to 5 × 106/mL. After 3 days of culture, half of the medium was replaced with fresh medium containing interleukin (IL)-2 (final concentration of 10 U/mL). On day 5, cells were harvested and tested in a standard 4-hour 51Cr release assay (26). Briefly, target AB12 cells (105/100 μL of PBS) were labeled with 100 μCi/mL of 51Cr solution for 1 hour and incubated with effector cells for 4 hours at different effector to target (E:T) ratios in triplicate, and 51Cr release was determined by analyzing the supernatants in a Microplate Scintillation Counter (Perkin Elmer). The percentage of specific lysis was calculated according to the formula: 100 × [(experimental release – spontaneous release)/(maximal release – spontaneous release)]. Spontaneous release and maximum release were obtained from wells containing target cells incubated in medium alone or in 2% acetic acid, respectively.

Combination treatment of tumor-bearing mice with CTLA-4 blocking antibody between cycles of chemotherapy

Mice were randomly divided into 4 groups as follows when tumor size reached 5 mm in diameter: (i) no treatment (NoRx); (ii) anti-CTLA-4 monoclonal antibody alone (anti-CTLA-4), 100 μg per mouse was injected i.p. once weekly for 3 doses, that is, on day 5, 12, and 19 after tumor cell injection; (iii) cisplatin alone (Cis), 5 mg/kg body weight was injected i.v. through the tail vein once weekly for 3 doses, that is, on day 4, 11, and 18 after tumor cell injection; (iv) combination therapy, anti-CTLA-4 monoclonal antibody (mAb) was given one day after each dose of cisplatin (Cis + anti-CTLA-4), thus following the same schedule as in groups 2 and 3.

Tumor size was measured twice weekly by a caliper, and tumor volume was estimated by a formula: \( V = \frac{ab^2\pi}{6} \), whereas \( a \) and \( b \) represent the longest and shortest maximal perpendicular diameters, respectively. Mice were euthanized when they met a predetermined tumor volume exceeded 350 mm3 to minimize pain and suffering and were scored as death. Survival time was evaluated from the day of tumor challenge to euthanization.

CD8 T-cell depletion

In a separate experiment, the tumor-bearing mice were treated with anti-CD8a mAb (clone: 53–6.7; eBioscience) 100 μg per injection to deplete CD8 T cells before administration of anti-CTLA-4 mAb to determine whether CD8 T-cell depletion could reverse the effect induced by CTLA-4 blockade.
Tumor cell repopulation was evaluated by immunohistochemical staining and flow cytometry for bromodeoxyuridine incorporation

On day 7 after the second dose of cisplatin, animals were sacrificed about 3 hours after bromodeoxyuridine (BrdU; Roche) 100 mg/kg body weight was injected i.p. Tumor tissues were removed at different time points after treatment and snap frozen immediately in liquid nitrogen, and then transferred to dry ice and kept at −80°C until frozen sectioning was conducted. Frozen sections were fixed in cold ethanol. Endogenous peroxidases, avidin and biotin were blocked using 1% hydrogen peroxide and the avidin/Biotin Blocking Kit (Dako). Sections were stained with a primary monoclonal antibody (1:50) against Ki67 or BrdU (eBioscience), and secondary anti-body linked to streptavidin–horseradish peroxidase (Dako). After washing, one Sigma FAST (D-4168) DAB (3,3’-diaminobenzidine) tablet and one urea hydrogen tablet (Sigma) were added to ddH2O to serve as a peroxidase substrate (125 µL/section) and slides were counter-stained with hematoxylin to visualize nuclei. Sections were then dehydrated and mounted with DPX (Ultra-mount, Scot Scientific).

Immunostained sections were quantified by using Aperio ImageScope digital scanner and Aperio ImageScope Viewer software version 9.0 (Vista, CA) under 200 magnification. Tumor cell repopulation was quantified as the proportion of Ki67 or BrdUrd-positive nuclear areas divided by total nuclear areas (26).

Single tumor cells were prepared by passing through the cell strainer (440 µm; BD Biosciences) and stained (1:50) with permeabilization buffer. BrdUrd-FITC antibody 1:30 was added and cells were exposed at room temperature in the dark for 30 minutes, then washed thrice for flow cytometry.

Immunohistochemistry and fluorescent immunostaining of tumor-infiltrating T cells

Tumor-infiltrating T cells were identified by primary rabbit anti-mouse CD3 (clone: SP7), rat anti-mouse CD4 (GK1.5), and CD8 (YTS169.4) monoclonal antibodies with 1:100 dilution (Abcam). Antibody–antibody reactions were visualized using DAB as the chromogen. For fluorescent immunostaining, sections were incubated with goat anti-rat or rabbit IgG (1:400 dilution). Purified rat or goat secondary antibodies, Alexa 488- or Alexa 568–labeled goat anti-rat or rabbit IgG (1:400 dilution). Purified rat or rabbit IgG was used as controls (Invitrogen). The detailed procedures were followed the instructions provided by the manufacturers.

Cell preparation and staining for flow cytometry

T-cell subsets were determined by using flow cytometry. On day 7 after the second dose of cisplatin, spleens and draining lymph nodes were removed from tumor-bearing mice and placed into ice-cold RPMI-1640 medium containing 1% FBS. The axillary lymph nodes from the tumor side were called draining lymph nodes. Peripheral blood was drawn from the heart of mice that were immediately euthanized by inhalation of CO2. Homogenized spleen and lymph node were passed through the cell strainer to achieve single cells. ACK lysis buffer (Invitrogen) was added and allowed to react for at least 15 minutes at room temperature to lyse red blood cells. After washing thrice with staining buffer, appropriate dilutions (1:50–1:100) of Abs or isotype controls were added to each tube, 15 minutes at room temperature in the dark. Staining of surface markers including CD3, CD4, CD8, and ICOS were washed thrice with staining buffer and resuspended in 1% paraformaldehyde/PBS (v/v; Sigma). After fixation with 1% paraformaldehyde at 4°C overnight, cells were permeabilized for intracellular staining of IFN-γ, perforin, and granzyme B and fixed with 200 µL Permeabilization/Fixation Solution (eBioscience) and then washed twice with permeabilization buffer. Anti-mouse antibodies against IFN-γ (1:30), perforin (1:50), and granzyme B (1:50) were added and maintained at room temperature for 20 minutes in the dark.

Single-cell suspensions were stained with monoclonal antibodies conjugated with different fluorescent dyes, CD3 (clone:17A2)-PerCP-Cy5.5, CD4 (clone:RM4-5)-FITC, CD8 (clone: H35-17.2)-APC, ICOS (clone: 7E.17G9)-PE, IFN-γ (clone:XMG1.2)-PE, perforin (clone:eBioOMAK-D)-FITC, and granzyme B (clone:16G6)-PE. All antibodies and isotypes were purchased from eBioscience or BioLegend. All cells from the same group were pooled together and considered to be as one sample for running flow cytometry. Becton Dickinson LSR II Flow Cytometer and FACSDiva software were used for data acquisition, and FlowJo software was used for analysis.

RNA extraction and real-time reverse transcription PCR

On day 7 after cisplatin treatment, spleens and tumor tissues were collected and total RNA was extracted from tumor tissues using TRIzol Reagent (Invitrogen), and RNeasy MinElute Cleanup Kit (QIAGEN) enabled cleanup of RNA. cDNA was synthesized with High-Capacity cDNA Reverse Transcription Kits (ABI) on a PTC-100 Programmable Thermal controller (MJ research Inc.) following the manufacturer’s protocols. Regular PCR was carried out to establish reverse transcription PCR (RT-PCR) standards of all target genes including CD3, CD4, CD8, ICOS, IL-2, IFN-γ, granzyme B, and perforin and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). DNA fragments were obtained from regular PCR on a PTC-100 Programmable Thermal. Regular PCR was carried out by 10× High Fidelity PCR Buffer, Platinum Taq polymerase High Fidelity, 50 mmol/L MgSO4, 10 mmol/L dNTP Mix (Invitrogen). A SYBR GREEN real-time PCR was carried out on ABI PRISM 7900HT system. PCR was composed of Power SYBR GREEN PCR 2× Master Mix (ABI), 200 mmol/L primer, and 2 µL 500 ng/µL cDNA × 40 cycles. Primers of all target genes and housekeeping gene were designed by using ABI Prism Primer Express software version 2.0.
Statistical analysis
All data are presented as the mean ± SEM. Statistical analyses were conducted using GraphPad Prism 5 statistical software. For all statistical analyses, a 2-tailed P value of less than 0.05 was considered to be statistically significant. Single-group data were assessed using unpaired Student t test. Tumor size, BrdUrd incorporation, and Ki67 proportion of positive nuclear areas, the expression of cytokine gene expression, T-cell subsets, among groups were analyzed by using one-way repeated measures ANOVA Newman–Keuls test for multiple comparisons. A value of P < 0.05 was considered significantly different for all comparisons. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Kaplan–Meier nonparametric regression analysis was conducted to assess the survival time of tumor-bearing animals with significance determined by the log-rank test.

Results
Murine mesothelioma AB12 is immunogenic
Specific cell lysis of cytotoxic T cells was observed in tumor-bearing mice compared with naive mice in the presence or absence of stimulation with tumor cell lysate (Fig. 1A). The production of representative cytotoxic
cytokine IFN-γ was confirmed to increase in CD8 T cells, whereas the CD4 T cells produced a fairly small amount of IFN-γ even though they were stimulated by tumor cell lysate (Fig. 1B).

The impact of CTLA-4 blockade on tumor growth at the early stage of tumor development in murine mesothelioma models and on T-cell activation

Administration of anti-CTLA-4 monoclonal antibody on day 1 and 3 after tumor cell injection resulted in dramatic growth delay in both AB12 and AC29 tumor models (Fig. 1C). Some tumors completely disappeared after treatment, and no tumor growth was observed in these tumor-free mice when they were rechallenged with the same tumor cell line. The absolute number of tumor-infiltrating CD4 and CD8 T cells after treatment increased in both models, AC29 is not shown (Fig. 1D).

In tumor-bearing mice, CTLA-4 blockade during chemotherapy delays tumor growth and improved survival, but antitumor effect induced by CTLA-4 blockade is reversed by CD8 T-cell depletion

Administration of anti-CTLA-4 mAbs during the intervals of cisplatin treatments (Cis + anti-CTLA-4) was significantly more effective than cisplatin alone (Cis), anti-CTLA-4 mAb alone (anti-CTLA-4), and untreated controls (NoRx). Tumor growth curves indicated that Cis + anti-CTLA-4 resulted in the best tumor growth delay in AB12 tumor model, even though cisplatin alone (Cis) resulted in significant growth delay. In contrast to the effect that was observed when CTLA-4 mAb was injected on days 1 and 3 after tumor cell injection, anti-CTLA-4 had only minor effect to delay the growth of AB12 tumors when it is given at 5 days after tumor challenge (Fig. 2A).

The blockade of CTLA-4 signaling not only enhanced antitumor activity, but also significantly prolonged survival of tumor-bearing mice, as shown in Fig. 2B. Median survival for the Cis + anti-CTLA-4 is 38 days, compared with Cis 30 days [P = 0.0139; HR, 14.62; 95% confidence interval (CI), 1.723–124.0], anti-CTLA-4 21 days (Cis + anti-CTLA-4 vs. anti-CTLA-4: P = 0.0477; HR, 0.5526; 95% CI, 0.3288–0.7764), and NoRx 18 days (P = 0.0050; HR, 0.4737; 95% CI, 0.2236–0.7238), respectively. The body weight of mice did not change significantly after completing treatment.

The antitumor effect induced by CTLA-4 blockade could be reversed by Cis8 T-cell depletion. Tumor growth was similar with the untreated mice (Fig. 2C).

Inhibition of tumor cell repopulation by administration of CTLA-4 blocking mAb during the courses of chemotherapy in tumor-bearing mice

Representative images of tumor sections stained with hematoxylin and eosin (H&E), BrdUrd, and Ki67 are shown in Fig. 3A. In the Cis + anti-CTLA-4–treated tumor, it is common to see the condensed nuclei and some areas replaced by infiltrating lymphocytes. Quantitative analysis of the immunostaining sections indicated that the percentage of positive nuclear staining for

![Figure 2. The effect of CTLA-4 blockade between the intervals of chemotherapy on tumor growth and survival. Treatment was initiated when tumors grew to a certain size. Cisplatin was injected i.v. once weekly for 3 doses, and anti-mouse CTLA-4 mAb was injected i.p. 1 day after each cycle of chemotherapy. Tumor size was recorded by measuring the 2 maximal perpendicular diameters twice weekly. A, tumor growth curves, n = 5 per group, and the experiment was repeated at least twice. Error bars indicate SEM. Cis + anti-CTLA-4 (n = 15) versus Cis (n = 25), P = 0.0351; versus anti-CTLA-4 (n = 15), P < 0.0001; versus NoRx (n = 25), P < 0.0001; Cis versus anti-CTLA-4, P = 0.0043; versus NoRx, P = 0.0001; anti-CTLA-4 versus NoRx, P = 0.0331. B, survival curves; mice were euthanized when tumor volume exceeded 350 mm3. Pooled results from 3 independent experiments are shown. Cis + anti-CTLA-4 (n = 15) versus Cis (n = 25), P < 0.0001; versus anti-CTLA-4 (n = 15), P = 0.0477; versus NoRx (n = 25), P = 0.0014; Cis versus anti-CTLA-4, P = 0.0351; versus NoRx, P = 0.0014; Cis + anti-CTLA-4 + anti-CD8, P = 0.0351. C, anti-CTLA-4 resulted in some tumor growth delay when treated at the early stage of tumor development, but the effect could be reversed by Cis8 T-cell depletion with anti-CD8 mAb.
BrdUrd or Ki67 in tumor cells was significantly lower after treatment with cisplatin combined with anti-CTLA-4 mAb than with cisplatin alone, anti-CTLA-4 alone, or untreated tumors (Fig. 3B and C). Flow cytometric data also showed that the BrdUrd-positive proportion of tumor cells dropped in the tumors treated with Cis + anti-CTLA-4 when compared those treated with cisplatin alone, anti-CTLA-4 alone, or no treatment (Fig. 3D).

**Infiltration of T cells in the tumor microenvironment after treatment with CTLA-4 blocking antibody following chemotherapy in tumor-bearing mice**

Tumor-infiltrating T cells were identified by CD3-specific immunostaining (Fig. 4A, top) and quantified by positive area divided by total cellular areas including T cells and tumor cells. The proportion of tumor-infiltrating T cells increased significantly when CTLA-4 blockade was combined to chemotherapy (Fig. 4B). Similar results were obtained from fluorescent immunostaining (Fig. 4A, middle and bottom). Flow cytometric results also showed that the absolute number and proportion of total T cells and CD4 and CD8 T cells rose after treatment with Cis + anti-CTLA-4 compared with other groups (Fig. 4C).

**Gene expression of T-cell activation markers and associated cytolytic cytokines in tumor-bearing mice**

The gene expression of T-cell activation markers such as ICOS increased in the tumors after treatment with Cis + anti-CTLA-4, compared with Cis alone, anti-CTLA-4 alone, or untreated tumors. More importantly, the genes of cytolytic cytokines and enzymes such as IL-2, IFN-γ,
Figure 4. A, infiltration of T cells in tumor microenvironment after treatment with anti-CTLA-4 following chemotherapy was determined by immunohistochemical staining for CD3 (top, × 200) and fluorescent immunostaining for CD3-green/CD4-red/DAPI-blue (middle, × 400), CD3-green/CD8-red/DAPI-blue (bottom, × 400), yellow color represents double positive of CD3/CD4 or CD3/CD8 T cells. B, quantitative analysis was conducted using an ImageScope software based on completion of entire section scanning of CD3 immunostaining. Combination of cisplatin and anti-CTLA-4 mAb resulted in a significant increase of CD3-positive proportion. Cis + anti-CTLA-4 versus NoRx, *P < 0.0001; Cis + anti-CTLA-4 versus Cis, *P = 0.0001, whereas anti-CTLA-4 versus NoRx, *P = 0.144, and Cis versus NoRx, *P = 0.0038. C, the number of tumor-infiltrating CD3, CD4, and CD8 T cells at 7 days after the second dose of cisplatin was analyzed using flow cytometry. DAPI, 4′,6-diamidino-2-phenylindole.

granzyme B, and perforin had higher expression levels in the Cis + anti-CTLA-4-treated tumors, as shown in Fig. 5. However, once tumor grew to a certain size, at 50 mm³ for example, monotherapy with CTLA-4 blockade did not result in significant growth delay of tumors or significant change of the gene expression.

Perforin and granzyme B from T cells infiltrated in tumor and draining lymph node

On day 7 after completing treatment, the expression of perforin and granzyme B in CD8⁺ T cells in tumor and the draining lymph node was assessed by flow cytometry. Cis + anti-CTLA-4 treatment resulted in an increase of granzyme B on CD8⁺ T cells in both tumor (Fig. 6A) and draining lymph node (Fig. 6B), especially in the draining lymph node, whereas perforin increased little in tumor or draining lymph node.

Discussion

CTLA-4 blockade offered significant therapeutic benefits to AB12 tumor-bearing mice when combined with chemotherapy. Administration of anti-CTLA-4 mAb during the intervals of cisplatin treatments slowed down tumor growth and prolonged survival of tumor-bearing mice. This effect was associated with significant inhibition of tumor cell repopulation between cycles of cisplatin treatments and significant increased in the total number of T cells and CD4⁺, CD8⁺ T cells infiltrating into the tumor. Anti-CTLA-4 combined with chemotherapy also resulted in an increased gene expression of IL-2, IFN-γ, granzyme B, perforin, and ICOS in the tumor, suggesting that the combination of chemotherapy and CTLA-4 in between cycles of chemotherapy enhanced the antitumor immune responses.

Because it is difficult to evaluate the antitumor effect by measuring tumor size in orthotopic models of intrapleural or intraperitoneal mesothelioma, we used a subcutaneous tumor model by injecting murine mesothelioma AB12 cells into the right flank of BALB/c mice. This model made it easier to evaluate the effect on tumor growth and also possible to study the impact of CTLA-4 blockade on cancer cell repopulation and the antitumoral immune response during the intervals between treatments (26). Combination chemotherapy with cisplatin and pemtrexed is currently administered as the first-line chemotherapy in the patients with advanced malignant pleural mesothelioma (27, 28), this combination treatment was able to prolong survival time by approximately 3 months, compared with cisplatin alone (29).
this subcutaneous mouse model have indicated that pemetrexed either alone or in combination with cisplatin did not result in significant benefit in controlling tumor growth (Supplementary Data, Supplementary Fig. 1S). Similar reports were also found in xenograft studies (30). Single-agent cisplatin was therefore selected as standard chemotherapy in our mouse model. The tumor-bearing mice were treated once weekly to observe the effect on tumor growth and survival. Our preliminary results indicated that tumor growth curves were clearly separated by weekly doses of chemotherapy (Supplementary Data, Supplementary Fig. 2S). Three weekly doses appeared to be most optimal for our mouse model, as this modality did not result in severe toxicity.

Therapies for mesothelioma are very limited so far (28, 29, 31). Therefore, immunotherapy such as targeting immunosuppressive checkpoints has raised a lot of interest for the treatment of this disease. CTLA-4 blocking antibody has recently been approved to treat metastatic melanoma by the U.S. Food and Drug Administration (32). This will open an avenue for treatment of other types of cancer. Even though limited studies have yet been carried out on mesothelioma, our current study shows that administration of anti-CTLA-4 therapy between cycles of chemotherapy results in tumor growth delay and improves survival, suggesting that anti-CTLA-4-based immunotherapy may be a potential therapy for the treatment of patients with malignant pleural mesothelioma.

At the early stage of tumor development, treatment with CTLA-4 blockade resulted in dramatic tumor growth delay and even tumor disappearance. However, this strategy is not feasible clinically as diagnosis is usually made at relatively late stages in patients with mesothelioma. Early treatment with CTLA-4 blockade led to an increase of tumor-infiltrating CD4+ and CD8+ T cells in both AB12 and AC29 tumor models. As shown in the chromium-release assay, the splenocytes derived from AB12 tumor-bearing mice had better cell killing than those from naive spleen through the production of more cytolytic cytokine IFN-γ (Fig. 1). Early treatment resulted in complete tumor disappearance in some mice, and rechallenge with same tumor cells did not lead to tumor growth. All the above evidence suggests that both AB12 and AC29 mesotheliomas are immunogenic and accounts for response to anti-CTLA-4 blockade-based immunotherapy. We selected the AB12 model in the series of experiments carried out on tumor-bearing mice because the subcutaneous tumor growth was more homogenous in AB12 than in AC29 mice.

The mechanisms of CTLA-4 signaling have been extensively studied (33). Other studies and ours showed that blockade of CTLA-4 is able to enhance antitumor response (34). However, the mechanisms by which CTLA-4 blockade enhances CD8 function in this context is not yet fully understood. We believe that the paradigm between Treg and IL-17–producing CD4+ T cells (T-helper cell 17, T17) and CD8+ T cells (Tc17) is of importance. Recent evidence suggests that the numbers of Treg and T17 cells are inversely correlated in the same tumor and that considerable functional plasticity exists between Treg and T17 cells (35). CTLA-4 induces
TH17 cells in peripheral blood of patients with metastatic melanoma and TH17 cells have been shown to promote antigen-specific antitumor immunity (36). Hence, the combination of chemotherapy and CTLA-4 blockade could skew the Treg/TH17 balance toward a TH17 phenotype. Although we have not measured the levels of Treg and TH17 in this series of experiments, we had previously shown in a similar subcutaneous model of mesothelioma that Treg blockade in combination with chemotherapy resulted in similar outcome with improved survival and increased cytotoxicity by CD8⁺ T cells in the tumor microenvironment (26). Preliminary results in our laboratory have also shown an increased level of IL-17 in the tumor microenvironment in the group of mice receiving CTLA-4 blockade in combination with chemotherapy (data not shown). Considering the importance of CD8⁺ T cells in mediating the CTLA-4 response, the recently described Tc17 cells could also be of importance in generating the response to CTLA-4 (37). Although Tc17 do not express granzyme B and perforin and are not able to mediate cell lysis in vitro, in vivo Tc17 can rapidly convert into an IFN-γ-secreting phenotype producing CD8⁺ T cells and mediate tumor rejection (37). Although the potential skewing of both CD8⁺ T cells and CD4⁺ T cells in the tumor microenvironment toward an IL-17–producing phenotype with the administration of CTLA-4 blocking antibody remains to be shown, better understanding of CTLA-4 in this context will likely help us refine cancer therapies.

CTLA-4 blockade is a promising cancer treatment and its effect can be potentiated when it is combined with conventional therapies, such as chemotherapy, radiotherapy, or cryotherapy as recently showed in our study and other preclinical work (38). In several phase I clinical trials including melanoma, ovarian, and prostate cancers as well as lymphoma, blockade of CTLA-4 resulted in tumor regression and was well tolerated, the main risk being the development of severe autoimmune adverse effects (39–42). In our animal study, there was no severe side effect using this treatment schema. Autoimmunity may be minimized by using sequential administration of the therapeutic antibody following each dose of cisplatin rather than concurrent administration. Surviving cancer cells may also be more effectively targeted by immunotherapy during the intervals of chemotherapy. Further studies are required to optimize the timing and scheduling of administration of immunotherapy and chemotherapy to maximize the synergistic effect.

Although the biology of mesothelioma is largely unknown, tumor progression is attributed to the balance of cell proliferation and lack of apoptotic cell death. Some investigators have observed that a majority of

Figure 6. Cytolytic enzymes were evaluated on day 7 after the second dose of cisplatin by flow cytometry. Intracellular staining was carried out for granzyme B and perforin from T cells infiltrated in tumor (A) and draining lymph node (B). The cells from 5 mice per group were pooled together.
malignant mesothelioma have high expression of inhibitor apoptosis proteins and low expression of proliferation markers such as Ki67 (43). However, the expression of these factors varied according to the anatomic site (peritoneum versus pleura) and the tumor microenvironment (effusion vs. solid tumor; ref. 43). In addition, the expression of proliferation marker such Ki67, although present in a small proportion of the tumor, was always expressed and the level of expression was more strongly associated with survival than inhibiting apoptosis proteins. Hence, these findings suggest that cell proliferation and dysregulation of apoptotic cell death are both present in mesothelial tumors and that proliferation factors may be of greater importance for survival, thus supporting the role of cell repopulation as a potential cause of clinical failure to chemotherapy in mesothelioma.

A number of studies, including our own and other work on mesothelioma, have shown the effect of chemotherapy on the development of an antitumor immune similarity. Similarly to our experience, Nowak and colleagues observed that gemcitabine increased cellular antitumor immunity through a CD8 T-cell-dependent pathway in a mouse model of mesothelioma (44). The resulting tumor-specific immune response is critical for the eradication of tumor cells that may survive therapy. Recent evidence suggests that cancer stem cells survive chemotherapy and/or radiotherapy and repopulate the tumor leading to treatment failure (45, 46). Hence, targeting stem cells may be of prime importance for long-term remission of the tumor. Immunotherapy may be a promising therapeutic option to target these cells and generate a long lasting memory response (47).

In conclusion, we have shown that CTLA-4 may play an important role in mesothelioma tolerance, and blockade of CTLA-4 signaling in combination with chemotherapy showed promising results that may be of importance for the development of new clinical trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
The authors thank Dr. Yidan Zhao for his assistance in statistical analysis.

Grant Support
This work was partly supported by the Mesothelioma Applied Research Foundation (MARF, USA) and the Mesothelioma Foundation at Princess Margaret Hospital (MPMH, Canada). Dr. M. de Perrot is the recipient for both grants.

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Received January 11, 2012; revised May 9, 2012; accepted May 9, 2012; published OnlineFirst May 14, 2012.

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Molecular Cancer Therapeutics

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