CTLA-4 blockade expands infiltrating T cells and inhibits cancer cell repopulation during the intervals of chemotherapy in murine mesothelioma

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Abbreviation list: CTLA-4: cytotoxic lymphocyte associated antigen-4; NoRx: no treatment; Cis: cisplatin; sc: subcutaneously; BrdU: bromodeoxyuridine; DAB: diaminobenzidine.

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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 might be able to inhibit cancer cell repopulation and enhanced the anti-tumoral 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 subcutaneous murine mesothelioma model. CTLA-4 blocking antibody was administered following each cycle of chemotherapy, and monotherapy was included as controls. Anti-tumor effect was evaluated by tumor growth delay and survival of the animals. Tumor cell repopulation was quantified by BrdU incorporation and Ki67 by immunohistochemistry and/or flow cytometry. In vitro cell killing was determined by classic chromium-released assay, and RT-PCR was performed to determine the gene expression of associated cytokines.

Anti-CTLA4 monoclonal antibody was able to inhibit tumor growth at early stage of tumor development. Anti-tumor 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 demonstrated that the gene expression of IL-2, IFN-\(\gamma\), granzyme B, and perforin increased in the tumor milieu.

Blockade of CTLA-4 signaling demonstrated effective anti-cancer 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.
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 two ligands (B7-1, B7-2) with its co-stimulatory 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 four molecules are perhaps the most important cofactors functioning in an immunological 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 anti-tumor immune responses and lead to durable anti-tumor 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 might 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 might be an important cause of clinical drug resistance. Therefore, inhibition of this process might be able to improve the efficacy of chemotherapy (24, 25).

Current evidence has demonstrated 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 cisplatin treatments in murine mesothelioma models to evaluate the benefit of antitumor effect.
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% fetal bovine serum 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 (2×10⁶) were injected subcutaneously (sc) into the right flank of female BALB/c and CBA/J mice which were provided by The Jackson Laboratory (Bar Harbor, Maine). 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 CTLA4 monoclonal antibody (Clone: 9H10, eBioscience, San Diego, CA) 100µg/dose by intraperitoneal (ip) injection on days 1 and 3 after tumor cell injection (ie, 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 naïve or AB12-bearing mice at 7 days after tumor challenge were pooled into 24-well plates at a concentration of 2.5-5×10^6/ml. After 3 days of culture, half of the medium was replaced with fresh medium containing IL-2 (final concentration of 10U/ml). On day 5, cells were harvested and tested in a standard 4-hour ^51^Cr release assay (26). Briefly, target AB12 cells (10^6/100µl of PBS) were labeled with 100µCi/ml of Cr^51^ solution for one hour and incubated with effector cells for 4h at different effector to target (E:T) ratios in triplicate, and ^51^Cr release was determined by analyzing the supernatants in a Microplate Scintillation Counter (Perkin Elmer, Waltham, MA). 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 four groups as follows when tumor size reached 5mm in diameter: 1) No treatment (NoRx); 2) Anti-CTLA4 monoclonal antibody alone (Anti-CTLA4), 100µg per mouse was injected ip once weekly for 3 doses, i.e. on day 5, 12 and 19 after tumor cell injection; 3) Cisplatin alone (Cis), 5mg/kg body weight was injected intravenously (iv) through the tail vein once weekly for 3 doses, i.e., on day 4, 11 and 18 after tumor cell injection; 4) Combination therapy, Anti-CTLA4 mAb was given one day after each dose of cisplatin (Cis+Anti-CTLA4), thus following the same schedule as in group 2 and 3.
Tumor size was measured twice weekly by a caliper, and tumor volume was estimated by a formula: $V = ab^2 \pi / 6$, whereas $a$ and $b$ represent the longest and shortest maximal perpendicular diameters, respectively. Mice were euthanized when they met a predetermined tumor volume exceeded 350mm$^3$ 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) 100µg/injection to deplete CD8 T cells before administration of anti-CTLA4 mAb in order to determine if 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 BrdU incorporation**

On day 7 after the 2$^{nd}$ dose of cisplatin, animals were sacrificed about 3 hours after bromodeoxyuridine (BrdU, Roche, Mannheim, Germany) 100mg/kg body weight was injected ip. 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 performed. 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, Carpinteria, CA). Sections were stained with a primary monoclonal antibody (1:50)
against Ki67 or BrdU (eBioscience), and secondary antibody linked to streptavidin-HRP (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 (125μl/section) and slides were counter-stained with hematoxylin to visualize nuclei. Sections were then dehydrated and mounted with DPX (Ultramount, 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 BrdU positive nuclear areas divided by total nuclear areas (26).

Single tumor cells were prepared by passing through the Cell Strainer (Φ40μm, BD Biosciences, San Diego, CA), and stained similarly as for intracellular cytokine IFN-γ staining. DNase I (Sigma) was applied after permeabilization. BrdU-FITC antibody 1:30 was added and cells were exposed at room temperature (RT) in the dark for 30min, 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, Cambridge, MA). Antigen-antibody reactions were visualized using diaminobenzidine (DAB) as the chromogen. For fluorescent immunostaining, sections were incubated with goat anti-rat secondary antibodies, Alexa 488 or Alexa 568 labelled goat anti-rat or rabbit IgG (1:400
Purified rat or rabbit IgG was used as controls (Invitrogen, Burlington, ON). 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 2nd dose of cisplatin, spleens and draining lymph nodes were removed from tumor-bearing mice and placed into ice-cold RPMI1640 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, Carlsbad, CA) was added and allowed to react for at least 15min at RT to lyse red blood cells. After washing thrice with staining buffer, appropriate dilutions (1:50~100) of Abs or isotype controls were added to each tube, 15min at RT in the dark. Staining of surface markers including CD3, CD4, CD8 and ICOS were washed thrice with staining buffer and resuspended in 1% paraformadehyde/PBS (v/v) (Sigma). After fixation with 1% paraformadehyde at 4°C overnight, cells were permeabilized for intracellular staining of IFN-γ, perforin and granzyme B and fixed with 200µl 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 RT for 20min 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, CD8β (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 (San Diego, CA). 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 (San Jose, CA) and FACSDiva™ software were used for data acquisition™ and FlowJo™ software was used for analysis.

RNA extraction and real-time reverse transcription-PCR (RT-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, Valencia, CA) enabled cleanup of RNA. cDNA was synthesized with High-Capacity cDNA Reverse Transcription kits (ABI, Foster City, CA) on a PTC-100™ Programmable Thermal controller (MJ research Inc., Gaithersburg, MD) following the manufacturer’s protocols. Regular PCR was carried out to establish RT-PCR standards of all target genes including CD3, CD4, CD8, ICOS, IL-2, IFN-γ, granzyme B, and perforin, and house-keeping gene GAPDH. DNA fragments were obtained from regular PCR on a PTC-100™ Programmable Thermal. Regular PCR was performed by 10× High Fidelity PCR Buffer, Platinum® Taq polymerase High Fidelity, 50mM MgSO₄, 10mM dNTP Mix (Invitrogen). A SYBR GREEN real-time PCR was performed on ABI PRISM 7900HT system. PCR was composed of Power SYBR® GREEN PCR 2× Master Mix (ABI), 200nM primer and 2µl 500ng/µl cDNA ×40 cycles. Primers of all target genes and house-keeping 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 performed using GraphPad Prism 5 statistical software (La Jolla, CA). For all statistical analyses, a two-tailed P value of less than 0.05 was considered to be statistically significant. Single-group data were assessed using unpaired Student's t test. Tumor size, BrdU 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 analysis of variance (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 performed 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 naïve 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-CTLA4 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 anti-tumor effect induced by CTLA-4 blockade is reversed by CD8 T cell depletion

Administration of anti-CTLA-4 mAb during the intervals of cisplatin treatments (Cis+Anti-CTLA4) was significantly more effective than cisplatin alone (Cis), anti-CTLA-4 mAb alone (Anti-CTLA4), and untreated controls (NoRx). Tumor growth curves indicated that Cis+Anti-CTLA4 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 day 1 and 3 after tumor cell injection, anti-CTLA4 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-CTLA4 is 38 days, compared with Cis 30 days (p=0.0139, hazard ratio (HR)=14.62; 95% CI of ratio: 1.723-124.0), Anti-CTLA4 21 days (Cis+Anti-CTLA4 vs Anti-CTLA4: p=0.0477, HR=0.5526, 95% CI of ratio: 0.3288-0.7764), and NoRx 18 days (p=0.0050, HR=0.4737; 95% CI of ratio: 0.2236-0.7238), respectively. Mice treated with Cis alone had longer survival than NoRx, P=0.0114, HR=0.6000; 95% CI of ratio: 0.3499-0.8501. The body weight of mice did not change significantly after completing treatment.

The anti-tumor effect induced by CTLA-4 blockade could be reversed by CD8 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 hemotoxylin and eosin (H&E), BrdU and Ki67 are shown in Fig. 3A. In the Cis+Anti-CTLA4 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 BrdU or Ki67 in tumor cells was significantly lower after treatment with cisplatin combined with anti-CTLA4 mAb than with cisplatin alone, anti-CTLA4 alone or untreated tumors (Fig. 3B & 3C). Flow Cytometry data also showed that the BrdU positive proportion of tumor cells dropped in the tumors treated with Cis-Anti-CTLA4 when compared those treated with cisplatin alone, anti-CTLA4 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 panel) 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 panels). Flow Cytometry results also demonstrated that the absolute number and proportion of total T cells and CD4 and CD8 T cells rose after treatment with Cis+Anti-CTLA4 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-CTLA4, compared with Cis alone, Anti-CTLA4 alone or untreated tumors. More importantly, the genes of cytolytic cytokines and enzymes such as IL-2, IFN-γ, granzyme B, and perforin had higher expression levels in the Cis+Anti-CTLA4-treated tumors, as shown in Fig. 5. However, once tumor grew to a certain size, at 50mm³ 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 infiltrated in tumor and the draining lymph node was assessed by flow Cytometry. Cis$\text{+}$.Anti-CTLA4 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-CTLA4 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-CTLA4 combined with chemotherapy also resulted in an increased gene expression of IL-2, IFN-$\gamma$, granzyme B, perforin and ICOS in the tumor, suggesting that the combination of chemotherapy and CTLA-4 in between cycles of chemotherapy enhanced the anti-tumor immune responses.

Since it is difficult to evaluate the anti-tumor effect by measuring tumor size in orthotopic models of intrapleural or intraperitoneal mesothelioma, we employed 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 anti-tumoral immune response during the intervals between treatments (26). Combination chemotherapy with cisplatin and pemetrexed is currently administered as the first line chemotherapy in the advanced MPM patients (27, 28), this combination treatment was able to prolong survival time by approximately 3 months, compared to cisplatin alone (29). Our \textit{in vivo} studies in 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 (\textit{Supplementary data, 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 (\textit{Supplementary data, Fig. 2s}). Three weekly doses appeared to be most optimal for our mouse model, since this modality did not result in severe toxicity.

Therapies for mesothelioma are very limited so far (28, 29, 31). Therefore, immunotherapy such as targeting immune suppressive 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 US 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 demonstrates that administration of anti-CTLA-4 therapy between cycles of chemotherapy results in tumor growth delay and improves survival, suggesting that anti-CTLA4-based immunotherapy might be a potential therapy for the treatment of MPM patients.
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 since 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 naïve spleen through the production of more cytolytic cytokine IFN-\(\gamma\) (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-CTLA4 blockade-based immunotherapy. We selected the AB12 model in the series of experiments performed 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 anti-tumor 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 (Th17) and CD8\(^+\) T cells (Tc17) is of importance. Recent evidence suggests that the numbers of Treg and Th17 cells are inversely correlated in the same tumor and that considerable functional plasticity exists between Treg and Th17 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 anti-tumor immunity (36). Hence, the combination of chemotherapy and CTLA-4
blockade could skew the Treg/Th17 balance towards 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+ 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 towards an IL-17 producing phenotype with the administration of CTLA-4 blocking antibody remains to be demonstrated, 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 demonstrated 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 might be minimized by utilizing
sequential administration of the therapeutic antibody following each dose of cisplatin rather than concurrent administration. Surviving cancer cells might 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 malignant mesothelioma have high expression of inhibitor apoptosis proteins and low expression of proliferation markers such as Ki-67 (43). However, the expression of these factors varied according to the anatomic site (peritoneum versus pleura) and the tumor microenvironment (effusion versus solid tumor) (43). In addition, the expression of proliferation marker such Ki-67, 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 anti-tumor immunity. 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 might play an important role in mesothelioma tolerance, and blockade of CTLA-4 signaling in combination with chemotherapy demonstrated promising results that may be of importance for the development of new clinical trials.
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References


**Figure legends**

**Fig. 1** Murine mesothelioma is immunogenic. The tumor responds to anti-CTLA4 therapy at the early stage of tumor development in murine mesothelioma models and T cell activation. A) On day 7 post tumor challenge, splenocytes were prepared from either tumor-bearing or naive mice and used for CTL assay as stated in methods. Splenocytes derived from AB12-bearing mice with or without stimulation of tumor cell lysate had more specific cell killing than those from naïve mice; B) Production of cytolytic cytokine IFN-γ was greater in the CTLs from AB12-bearing mice than naïve mice. C) On day 1 and 3 after tumor cell injection, mice were treated with anti-CTLA4 mAb, as indicated by the arrows. Anti-mouse CTLA-4 mAb resulted in tumor growth delay in both AB12 and AC29 tumor models when compared with controls; D) On day 7 post treatment with anti-CTLA4 mAb, the absolute number of tumor-infiltrating CD4 and CD8 T cells increased.

**Fig. 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 iv once weekly for 3 doses, and anti-mouse CTLA4 mAb was injected ip one day after each cycle of chemotherapy. Tumor size was recorded by measuring the two 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-CTLA4 (*n*=15) vs Cis (*n*=25): *p*=0.0351, vs Anti-CTLA4 (*n*=15): *p*<0.0001, vs NoRx (*n*=25): *p*<0.0001; Cis vs Anti-CTLA4: *p*=0.0043, vs NoRx: *p*<0.0001, Anti-CTLA4 vs NoRx: *p*=0.0331; B) Survival curves, mice were euthanized when tumor volume exceeded 350 mm³. Pooled results from three independent experiments are shown. Cis+Anti-CTLA4 (*n*=15) vs Cis (*n*=25): *p*<0.0001, vs Anti-CTLA4.
(n=12): p<0.0001, vs NoRx (n=25): p<0.0001; Cis vs Anti-CTLA4: p<0.001, vs NoRx: p<0.0001, Anti-CTLA4 vs NoRx: p<0.05; C) Anti-CTLA4 resulted in some tumor growth delay when treated at the early stage of tumor development, but the effect could be reversed by CD8 T cell depletion with anti-CD8 mAb.

**Fig. 3** Cancer cell repopulation between cycles of chemotherapy is inhibited by anti-CTLA-4 therapy. Tumor histology was shown in A) H&E staining, BrdU incorporation and Ki67 staining; B&C) Quantification of BrdU incorporation and Ki67 immunostaining was performed using Aperio ImageScope software to calculate the proportion of positive nuclear area occupied by BrdU or Ki67 divided by total nuclear areas; *, P<0.05; **, P<0.01; ***, P<0.001; D) BrdU incorporation in tumor cells was shown as the percentage of intracellular positive staining determined by flow Cytometry. Tumor cells from five tumors per group were pooled together. The experiment was repeated twice.

**Fig. 4** A) Infiltration of T cells in tumor microenvironment after treatment with anti-CTLA4 following chemotherapy was determined by immunohistochemical staining for CD3 (Top panel, 200×), and fluorescent immunostaining for CD3-green/CD4-red/DAPI-blue (Middle panel, 400×), CD3-green/CD8-red/DAPI-blue (Bottom panel, 400×), yellow color represents double positive of CD3/CD4 or CD3/CD8 T cells; B) Quantitative analysis was performed using an ImageScope software, based on completion of entire section scanning of CD3 immunostaining. Combination of cisplatin and anti-CTLA4 mAb resulted in a significant increase of CD3 positive proportion. Cis+Anti-CTLA4 versus NoRx p<0.0001, Cis+Anti-CTLA4 vs Cis: p=0.0001,
whereas Anti-CTLA4 vs NoRx p=0.144 and Cis vs NoRx p=0.6369; C). The number of tumor-infiltrating CD3, CD4 and CD8 T cells at 7 days after the 2\textsuperscript{nd} dose of cisplatin was analyzed using flow Cytometry.

**Fig. 5** Gene expression of T cell activation marker and associated cytolytic cytokines was determined by RT-PCR. Total RNA was extracted from the snap-frozen tumors of each group to quantify the expression level of CD3, CD4, CD8, IL-2, ICOS, IFN-\(\gamma\), granzyme B, and perforin in tumor. The details are described in the methods.

**Fig. 6** Cytolytic enzymes were evaluated on day 7 after the 2\textsuperscript{nd} dose of cisplatin by flow Cytometry. Intracellular staining was performed for granzyme B and perforin from T cells infiltrated in tumor (A), and draining lymph node (B). The cells from five mice per group were pooled together.
Figure 1

A

Splenocytes

5 days

IL-2

A B

AB12 lysate

Effector CTLs

NoRx

Tumor cells

(Cr 51)

Anti-CTLA4

Specific cell killing

E:T ratio

cell lys (%)

0 10 20 30 40 50

Sn

Sn+Lys

St

St+Lys

0.91

3.39

8.80

34.7

B

CD4+IFNg

CD4+IFNg

CD8+IFNg

CD8+IFNg

AC29 tumor

NoRx

Anti-CTLA4

days after tumor cell injection

0 5 10 15 20 25 30

0 100 200 300

AB12 tumor

NoRx

Anti-CTLA4

days after tumor cell injection

0 5 10 15 20 25

0 100 200 300

CD4+ T cells in tumor

NoRx

Anti-CTLA4

p=0.04

CD8+ T cells in tumor

NoRx

Anti-CTLA4

p=0.03
Figure 2

A. Graph showing tumor size (mm$^3$) over days after tumor challenge for different treatment groups: NoRx, Anti-CTLA4, Cis, and Cis+Anti-CTLA4. NS indicates no significant difference. Asterisks indicate statistical significance. 

B. Graph showing percent survival over days after tumor challenge for the same treatment groups as in A. 

C. Graph showing tumor size (mm$^3$) over days after tumor challenge for treatment groups: NoRx, Anti-CTLA4, Anti-CD8, and Anti-CTLA4+Anti-CD8.
Figure 3

A

H&E

Ki67

BrdU

NoRx

Anti-CTLA4

Cis

Cis+Anti-CTLA4

B

BrdU

C

Ki67

D

BrdU

NoRx

Anti-CTLA4

Cis

Cis+Anti-CTLA4
Figure 5
Figure 6

**A**

Perforin+ CD8 T cells in tumor

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Granzyme B+ CD8 T cells in tumor

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**B**

CD8

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Granzyme B

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Molecular Cancer Therapeutics

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Licun Wu, Zhihong Yun, Tetsuzo Tagawa, et al.

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