AMD3100 Augments the Efficacy of Mesothelin-Targeted, Immune-Activating VIC-008 in Mesothelioma by Modulating Intratumoral Immunosuppression

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Abstract

AMD3100 (plerixafor), a CXCR4 antagonist, has been demonstrated to suppress tumor growth and modulate intratumoral T-cell trafficking. However, the effect of AMD3100 on immunomodulation remains elusive. Here, we explored immunomodulation and antitumor efficacy of AMD3100 in combination with a previously developed mesothelin-targeted, immune-activating fusion protein, VIC-008, in two syngeneic, orthotopic models of malignant mesothelioma in immunocompetent mice. We showed that combination therapy significantly suppressed tumor growth and prolonged animal survival in two mouse models. Tumor control and survival benefit were associated with enhanced antitumor immunity. VIC-008 augmented mesothelin-specific CD8+ T-cell responses in the spleen and lymph nodes and facilitated intratumoral lymphocytic infiltration. However, VIC-008 treatment was associated with increased programmed cell death protein-1 (PD-1) expression on intratumoral CD8+ T cells, likely due to high CXCL12 in the tumor microenvironment.

Introduction

Malignant mesothelioma is an aggressive tumor that arises from the pleural and peritoneal mesothelium. Malignant mesothelioma is largely refractory to conventional therapies, and the median survival after symptom onset is often less than 12 months (1–4). Surgery, radiotherapy, and chemotherapy have improved quality of life but have made little impact on survival with this tumor. Studies over the last two decades suggest that immunotherapy may be a very promising avenue for the treatment of malignant mesothelioma, given that the tumor expresses antigens that can be targeted by the immune system (5, 6). Although clinical trials of various immunotherapeutic modalities have yielded significant benefit in certain cancers, including melanoma and non–small cell lung cancer (7), limitations in the efficacy of these therapeutic approaches have been observed and can be understood in light of the complexities of intratumoral immune dysregulation (8), which require the development of more efficacious combination immunotherapies that address these immune evasion mechanisms.

We previously described a fusion protein consisting of the broadly immune-activating Mycobacterium tuberculosis–derived heat shock protein 70 (MtbHsp70) and the tumor antigen–targeting activity of a single-chain variable fragment (scFv)-binding mesothelin (MSLN; ref. 9), a validated immunotherapy target (10). We evaluated the antitumor efficacy of this MSLN-targeted fusion protein as an in vivo vaccination strategy in syngeneic, immunocompetent mouse models of ovarian cancer and mesothelioma and demonstrated that this bifunctional fusion protein significantly enhances survival and slows tumor growth through the augmentation of tumor-specific, cell-mediated immune responses (9). In vivo CD8+ T-cell depletion studies demonstrated that this protective antitumor effect is mediated by

AMD3100 alone and in combination with VIC-008 modulated immunosuppression in tumors and the immune system through suppression of PD-1 expression on CD8+ T cells and conversion of regulatory T cells (Treg) into CD4+ CD25+ IL2+ CD40L+ helper-like cells. In mechanistic studies, we demonstrated that AMD3100-driven Treg reprogramming required T cell receptor (TCR) activation and was associated with loss of PTEN due to oxidative inactivation. The combination of VIC-008 augmentation of tumor-specific CD8+ T-cell responses with AMD3100 abrogation of immunosuppression conferred significant benefits for tumor control and animal survival. These data provide new mechanistic insight into AMD3100-mediated immunomodulation and highlight the enhanced antitumor effect of AMD3100 in combination with a tumor antigen–targeted therapy in mouse malignant mesothelioma, which could be clinically relevant to patients with this difficult-to-treat disease. Cancer Immunol Res, 6(5), 539–51. ©2018 AACR.
tumor-specific CD8+ T cells (9). The current version of this fusion protein, VIC-008, was derived from the original by modifications that remove redundant amino acids and introduce a single amino acid mutation, phenylalanine to valine, at position 381 of MtbHsp70 to prevent nonspecific peptide binding and presentation while retaining the immune-stimulatory capacity of the original protein. This fusion protein showed significantly improved efficacy in tumor control and animal survival in a mouse model of MSLN-expressing ovarian cancer over the original protein (13). However, the intratumoral immunosuppressive microenvironment, including, most notably, the presence of regulatory T cells (Tregs), may limit the survival in a mouse model of MSLN-expressing ovarian cancer (14).

The critical role of chemokine receptor 4 (CXCR4) and its ligand (CXCL12) in the pathogenesis of many tumors has been well-recognized (15, 16). AMD3100, a specific antagonist for CXCR4, was originally developed as an anti-HIV drug (17) and later applied as a reagent to mobilize hematopoietic stem cells from bone marrow (18). A number of studies have shown that AMD3100 can affect tumor growth, metastasis, and angiogenesis by blockade of the CXCL12/CXCR4 axis (19–21) and subsequent inhibition of PI3K-Akt or Ras/Raf/Erk1/2 signaling (22). We previously reported that the blockade of the CXCL12/CXCR4 axis with AMD3100 as a monotherapy in ovarian tumor–bearing mice conferred a survival advantage and elicited multimodal effects on tumor pathogenesis and intratumoral T-cell trafficking, including selective reduction of Tregs in the tumor (23).

However, the precise mechanism of AMD3100-mediated immunomodulation remains unknown.

Immunomodulators have been widely used in combination with tumor vaccines or immunotherapies for improving antitumor immune responses, which include removing or inhibiting suppressive cells such as Tregs, regulatory type II NKT cells, or myeloid-derived suppressor cells (24–29). In this study, we evaluated the antitumor efficacy of AMD3100 and VIC-008 in two syngeneic orthotopic models of malignant mesothelioma in immunocompetent mice and found that AMD3100 treatment enhanced the effects of VIC-008 in tumor control and animal survival. This response was associated with AMD3100-mediated neutralization of intratumoral immunosuppression. We also explored and revealed a mechanism by which AMD3100 modulates immunity alone and in combination with VIC-008 in murine models of malignant mesothelioma.

Materials and Methods

Reagents

The fusion protein VIC-008 was constructed with V8 and V1 from anti-MSLN p4 scFv fused to full-length MtbHsp70 with a (G4S)3 linker in between and a single amino acid mutation, phenylalanine in place of valine, at position 381 of MtbHsp70, which has been shown in our previous study (13). Proteins were expressed by WuXi Biologics in CHO cells and provided at a purity of above 95% by HPLC and an endotoxin level less than 1.0 EU/mg. AMD3100 was purchased from Abcam (#ab120718).

Tumor cells

40L and AE17 mouse mesothelioma cell lines were kind gifts from Dr. Agnes Kane (Department of Pathology and Laboratory Medicine, Brown University, Providence, RI). The cell lines were authenticated by morphology check under microscope. Cells were cultured at 37°C in DMEM (HyClone) supplemented with 1% l-glutamine, 1% penicillin-streptomycin, and 10% FBS (all from Gibco). Mycoplasma detection was performed before use by a Mycoplasma detection kit (Thermo Fisher; #4460626). Cells in the logarithmic phase were used for further assays.

Animal models

Five-week-old female C57BL/6 mice were obtained from the Jackson Laboratory and maintained in the gnotobiotic animal facility of Massachusetts General Hospital (MGH) in compliance with institutional guidelines and policies. After 1-week acclimatization, tumors were initiated with 4 × 10^6 40L cells or 2 × 10^6 AE17 cells per mouse administered i.p. A subset of the mice from each group were euthanized with i.p. administration of ketamine (9 mg/ml in saline) and xylazine (0.9 mg/ml in saline; Sigma) 7 days after the last treatment, and samples were harvested for immune profiling of tumors, inguinal and axillary lymph nodes, and spleens (processing described below).

The remaining animals in each group were monitored for survival. For survival studies, we observed the mice daily after inoculation of tumor cells. Tumor generation was consistently first evident via the appearance of abdominal distension, secondary to malignant ascites, and tumor-bearing mice were euthanized using carbon dioxide at the endpoint when signs of distress, including fur ruffling, rapid respiratory rate, hunched posture, reduced activity, and progressive ascites formation, were observed.

Splenocytes from T-Red/FoxP3-GFP transgenic mice, which were generated by Dr. Thorsten Mempel and were a kind gift from Dr. Gilles Benichou at MGH, were used as a source of fluorescently tagged Tregs by cell sorting, as described below. T-Red/FoxP3-GFP transgenic mice were generated by induction of uniform and selective expression of ds-RedII in all T cells and FoxP3-GFP. The method consists of inserting the red fluorescent protein ds-RedII under the control of the CD4 promoter and proximal enhancer. This construct lacks the intronic silencer, which prevents CD4 promoter activity in CD8+ cells. The mice were then fully backcrossed with FoxP3-GFP mice from the Jackson Laboratory. The FoxP3 construct expresses GFP under control of the forehead box p3 (FoxP3) promoter. The construct is designed such that no FoxP3 protein is produced, preventing overexpression. All animal studies were approved by the Institutional Animal Care and Use Committee of MGH.

Treatment

Beginning 7 days after tumor inoculation, treatments were administered by i.p. injection once a week for 4 successive weeks. VIC-008 was administered i.p. at 20 μg in 100 μl of saline per mouse once a week, and AMD3100 was given once a week by i.p. injection at 1 mg/kg of body weight in 100 μl of saline.

Immune profiling by flow cytometry

Tumors harvested at day 7 after the last treatment were mechanically disaggregated using sterile razor blades and digested at 37°C for 2 hours in RPMI 1640 with collagenase type IV for 40L tumors or type I for AE17 tumors (2 mg/ml; Sigma), DNase (0.1 mg/ml; Sigma), hyaluronidase (0.1 mg/ml; Sigma), and BSA (2 mg/ml; Sigma). Cell suspensions were passed through 100 μm filters to

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remove aggregates. Lymph nodes and spleens were mashed by two microslides and filtered through 40 μm strainers. Cells were washed with staining buffer (#420201; BioLegend) and stained with the conjugated antibodies for surface markers. In brief, 1 × 10^6 cells from lymph nodes or spleens or up to 1 × 10^7 cells from tumors were resuspended in staining buffer and distributed 100 μL/tube of suspension into plastic tubes. Fc receptors were blocked by anti-mouse CD16/32 (BioLegend; #101319). Conjugated primary antibodies were added and incubated on ice for 20 minutes in dark. Total live cells were determined by LIVE/DEAD staining (Thermo Fisher; #L23105).

For intracellular cytokine detection, after staining of surface markers, cells were fixed and permeabilized with fixation/permeabilization reagents from BioLegend (#424401) or eBioscience (#00-5521-00) and stained with the conjugated antibodies for intracellular markers.

Conjugated antibodies from eBioscience were as follows: PerCP-Cy5.5 CD40L (clone MR1) and APC-Cy7 PD-1 (clone J43). The following conjugated antibodies were purchased from BioLegend: Brilliant Violet 421 CD3 (clone 17A2), Brilliant Violet 711 CD4 (clone GK1.5), PE-Cy7 CD8a (clone 53-6.7), Alexa Fluor 488 Foxp3 (clone MF-14), Brilliant Violet 605 IL2 (clone JS65-5H4), and Alexa Fluor 488 IFNγ (clone XMG1.2). APC CD25 (clone PC61) and PE-PFN (clone A2B5) antibodies were purchased from BD Biosciences.

Flow cytometric analyses were performed using BD LSRFortessa X-20 (BD Biosciences). Gating strategies were determined by the Fluorescence Minus One. Flow data were analyzed by FlowJo V10 (TreeStar).

**Ex vivo culturing of splenocytes and cytokine detection**

Splenocytes were treated with red blood cell lysis buffer (BioLegend; #420301). Note that 2 × 10^6 splenocytes per well were plated in 24-well plates in RPMI 1640 medium supplemented with 1% L-glutamine and stimulated with recombinant mouse MSLN (2 μg/mL; BioLegend; #594006) for 72 hours. Brefeldin A and monesin (BioLegend; #420601 and #420701, respectively) were added into the culture medium during the last 5 hours. Splenocytes were then harvested, and intracellular cytokine staining was performed using anti-IFNγ antibodies for cytometric analysis.

**Ex vivo treatment of intratumoral CD3+CD8+ T cells by AMD3100**

In untreated AE17 tumor-bearing mice, tumors were harvested and digested (as described above). The intratumoral CD3+CD8+ cells were sorted out by a FACSARia (BD Biosciences) and then treated with AMD3100 (5 μg/mL) for 24 hours. After treatment, the cells were harvested, blocked by anti-CD16/32, then stained with conjugated antibodies specific for CD3, CD8, and programmed cell death protein-1 (PD-1; as described above), and analyzed by flow cytometry.

**In vitro reprogramming of Tregs**

T-Red/FoxP3-GFP transgenic mice express GFP in Foxp3+ Tregs. Spleens were collected from these mice and mashed and filtered through 40 μm strainers. Cells expressing GFP-Foxp3 from CD4+ splenocytes were sorted on a FACSARia (BD Biosciences) and then exposed to AMD3100 (5 μg/mL) in the presence or absence of anti-CD3/CD28 antibody (1 μg/mL) for 24 hours. Brefeldin A and monesin (BioLegend; #420601 and #420701, respectively) were added into the culture medium during the last 5 hours. The cells were then harvested and stained with conjugated antibodies specific for CD3, CD4, CD25, Foxp3, IL2, CD40L, and PTEN and analyzed by flow cytometry.

**Enzyme-linked immunosorbent assay**

Tumors, spleens, and inguinal and axillary lymph nodes were collected and then mashed with the help of liquid nitrogen for protein extraction. Tissue CXCL12 was evaluated by an ELISA kit (R&D Systems; #DY460 and #DY008), following the manufacturer’s instructions. For tissue CXCL12 quantification, 20 μg of total protein from each sample was added in each well of 96-well plates.

**Western blot**

For analyses of protein expression, sorted Tregs were lysed and run in nonreducing conditions or reducing conditions by adding reducing agent (Thermo Fisher; #NP004) and antioxidant (Thermo Fisher; #NP0005). The concentration of protein was tested using the BCA Protein Assay Kit (Thermo Fisher; #23227). Note that 40 μg total protein per sample was used for analysis. Before loading into the gels, lysates were mixed with loading buffer (Thermo Fisher; #NP0007) and heated in 70°C for 10 minutes. Cell lysates were then separated by Bis-Tris protein gels (Thermo Fisher; #NP00321) in running buffer (Thermo Fisher; #NP0002), transferred to methanol-soaked PVDF membranes (Thermo Fisher; #LC2002) in transfer buffer (Thermo Fisher; #NP0006), blocked by 5% BSA (for antibodies from Cell Signaling Technology; Sigma, #A7066) or 5% nonfat milk (for antibodies from Abcam and Santa Cruz Biotechnology) at room temperature for 1 hour, and immunoblotted with primary antibodies at 4°C overnight. Tris-buffered saline with 0.1% Tween-20 (Sigma; #P9416; TBST) was used as washing buffer in the whole procedure, followed by incubation with appropriate horseradish peroxidase–conjugated secondary antibody (Abcam; #ab79051 and #ab6741) at room temperature for 1 hour, and detection of the blots was performed by the ECL detection system (Chemidoc XRS+ imaging system; Bio-Rad). Antibodies against β-tubulin (#ab6046) and PTEN (#ab32199) were purchased from Abcam.

**Statistical analyses**

P values were calculated by GraphPad Prism 6. The P values for comparison among groups were obtained by one-way ANOVA with Dunnett multiple comparisons test or unpaired t test with Welch correction. The Kaplan–Meier method and log-rank test were used to compare survival among groups. P < 0.05 was considered statistically significant. Data are presented as mean ± SEM.

**Results**

Combination of AMD3100 and VIC-008 augments tumor control and mouse survival

We established two i.p. malignant mesothelioma models in immunocompetent C57BL/6 mice, separately using the syngeneic 40L and AE17 cell lines (30). Here, we tested the effect of AMD3100 and VIC-008, used alone or in combination, on tumor growth and animal survival in mesothelioma-bearing mice. In animals treated with VIC-008 alone (20 μg per mouse), the total weight of i.p. tumors collected 1 week after the last treatment was not significantly reduced (Fig. 1A and B), but animal survival was...
significantly prolonged (Fig. 1C and D) compared with saline control treatment in both the 40L and AE17 models ($P < 0.01$ and $P < 0.01$, respectively). AMD3100 alone at 1 mg/kg of mouse body weight did not confer significant benefit to survival in both 40L and AE17 mouse malignant mesothelioma models compared with saline control treatment. However, the combination treatment with VIC-008 and AMD3100 significantly enhanced tumor control ($P < 0.0001$ and $P < 0.001$, respectively) and prolonged animal survival ($P < 0.0001$ and $P < 0.001$, respectively) compared with saline control in both 40L and AE17 models. The combination treatment showed further significantly improved antitumor efficacy on inhibition of tumor growth ($P < 0.001$ and $P < 0.05$, respectively) and prolongation of mouse survival ($P < 0.0001$ and $P < 0.001$, respectively) compared with VIC-008 monotherapy in both 40L and AE17 models. These data indicate that when combined, AMD3100 significantly enhances the antitumor effect of VIC-008 in both 40L and AE17 malignant mesothelioma mouse models compared with monotherapy with either agent.

**VIC-008 increases lymphocyte infiltration in spleens, lymph nodes, and tumors**

Spleens, axillary and inguinal lymph nodes, and i.p. tumors were collected from tumor-bearing mice 1 week after the last treatment. Single cells were prepared from these tissues and analyzed by flow cytometry (Fig. 2A). The proportion of CD8$^+$ T cells in the total live cells recovered from spleens ($P < 0.05$ and $P < 0.05$, respectively) and tumors ($P < 0.01$ and $P < 0.01$, respectively) for both the 40L and AE17 models, and from lymph nodes ($P < 0.01$) in AE17 model, was significantly increased in the VIC-008 treatment group compared with that in the saline control group (Fig. 2B–F). In the VIC-008 and AMD3100 combination treatment group, the proportion of CD8$^+$ T cells in the total live cells recovered from spleens ($P < 0.05$ and $P < 0.05$, respectively) and tumors ($P < 0.01$ and $P < 0.05$, respectively) for both the 40L and AE17 models, and from lymph nodes ($P < 0.001$) in AE17 model were significantly increased compared with that in the saline control group, and AMD3100 treatment did not increase the proportion of CD8$^+$ T cells in these tissues. However, no difference in the proportion of CD8$^+$ T cells between the VIC-008 and combination treatment groups was seen, indicating that VIC-008 increased lymphocyte infiltration of these tissues.

**VIC-008 enhances tumor antigen-specific CD8$^+$ T-cell responses**

We next restimulated single cells isolated from the spleens and lymph nodes of tumor-bearing mice with recombinant MSLN ex vivo and analyzed intracellular IFN$\gamma$ in CD8$^+$ T cells. MSLN-specific IFN$\gamma$ expressions in splenic CD8$^+$ T cells, both in 40L ($P < 0.001$) and AE17 ($P < 0.001$) models, and in lymph node CD8$^+$ T cells in the AE17 model ($P < 0.01$) were significantly greater in mice treated with VIC-008 alone compared with that in mice treated with saline (Fig. 3). In VIC-008 and AMD3100

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**Figure 1.**

Tumor growth and survival in different treatment groups. All i.p. tumors were collected at day 7 after the last treatment and weighed in (A) 40L ($n = 10$ per group) and (B) AE17 ($n = 10$ per group) mouse malignant mesothelioma models. The survival of both the 40L and AE17 tumor-bearing mice. Numbers on the x-axis represent days of mouse survival after inoculation of tumor cells. NS, not significant; *, $P < 0.05$; ***, $P < 0.01$; ****, $P < 0.001$; and *****, $P < 0.0001$. Data were presented as mean ± SEM, representative of three independent experiments. Statistical method: (A and B) One-way ANOVA with Dunnett multiple comparisons and (C and D) Kaplan–Meier method and log-rank test.
combination treatment group. MSLN-specific IFNγ expressions in splenic CD8+ T cells, both in 40L (P < 0.01) and AE17 (P < 0.01) models, and in lymph node CD8+ T cells in the AE17 model (P < 0.001) were significantly greater compared with that in mice treated with saline. AMD3100 treatment by itself did not enhance antigen-specific IFNγ secretion in CD8+ T cells. No difference in the IFNγ expression in CD8+ T cells between the VIC-008 and combination treatment groups was seen. Together, these data support the view that VIC-008 treatment enhanced antitumor CD8+ T-cell responses in both the 40L and AE17 mesothelioma mouse models.

AMD3100 decreases PD-1 expression on CD8+ T cells

We next evaluated expression of PD-1 on CD8+ T cells in spleen, tumor, and lymph nodes. No significant difference between the VIC-008 treatment group and the saline control group in the proportion of PD-1–expressing CD8+ T cells in spleens in both the 40L and AE17 tumor–bearing mice and in lymph nodes for AE17 mice was seen (Fig. 4A–C). In contrast, significantly more intratumoral CD8+ T cells in the VIC-008–treated group expressed PD-1 in the 40L tumors (P < 0.05) and AE17 tumors (P < 0.01) compared with saline-treated controls and AMD3100 treatment (Fig. 4D and E). The percentage of PD-
1–expressing CD8+ T cells ranged between 43%–76% and 28%–47% in the 40L tumors and AE17 tumors, respectively, compared with only 5% to 10% in spleen and lymph nodes. These data indicate that the antitumor activity of the CD8+ T cells in the tumor environment induced by VIC-008 treatment could be obstructed by activation of the PD-1/PD-L1 pathway. Similarly, we found that significantly more intratumoral CD4+ T cells in VIC-008–treated mice expressed PD-1 in the 40L tumors (P < 0.01) and AE17 tumors (P < 0.05) compared with saline-treated controls and AMD3100 treatment (Fig. 4F and G). Given that others have reported the impact of CXCL12 on PD-1 expression (22, 31, 32), we examined the levels of CXCL12 in the in vivo intratumoral microenvironment. We found that the concentration of intratumoral CXCL12 measured by ELISA was significantly higher in VIC-008–treated AE17 tumor–bearing mice (2,962 ± 351 pg/mg) than that in saline-treated (1,444 ± 261 pg/mg; P < 0.01) and AMD3100–treated mice (1,044 ± 172 pg/mg; P < 0.01; Fig. 4H). In the VIC-008–treated mice, the concentration of CXCL12 was significantly higher in tumors than in spleens (P < 0.001) and lymph nodes (P < 0.001; Fig. 4I). These data indicated that high CXCL12 in intratumoral microenvironment may be associated with the upregulation of PD-1 expression on tumor-infiltrating T cells.

In concert with this, we found that AMD3100 reduced PD-1 expression on CD8+ T cells. AMD3100 treatment alone led to significantly fewer PD-1–expressing CD8+ T cells in spleens (P < 0.05 and P < 0.01, respectively) and tumors (P < 0.05 and P < 0.05, respectively) in both the 40L and AE17 tumor–bearing mice and in lymph nodes (P < 0.01) for AE17 mice compared with the saline-treated controls. No significant difference in the proportion of PD-1–expressing CD8+ T cells in these tissues between the AMD3100 monotherapy and AMD3100–VIC-008 combination therapy groups was found. In contrast, we did not observe significant inhibition of PD-1 on CD4+ T cells by AMD3100 in tumors (Fig. 4F and G).

In order to address whether AMD3100–driven PD-1 down-regulation on tumor-infiltrating CD8+ T cells could be mediated in isolated single cells, CD3+CD8+ T cells were sorted from tumor-infiltrating lymphocytes in untreated AE17 tumor–bearing mice and exposed in vitro to AMD3100 (5 μg/mL). AMD3100 significantly decreased the expression of PD-1 on CD8+ T cells (P < 0.0001; Fig. 4J and K). Together, these data showed that AMD3100 can inhibit CD8+ T cells from expressing PD-1 in spleens, lymph nodes, and tumors in vitro and mediate the downregulation of PD-1 on CD8+ T cells in vitro at the single-cell level. These data are consistent with previous studies that support the view that the CXCR4/CXCL12 axis and its antagonism modulate intratumoral immune checkpoint molecule expression (22, 31, 32).

**AMD3100 reduces tumor-infiltrating Treg**

We next evaluated the impact of AMD3100 on Treg. AMD3100 did not alter the proportion of Treg in the spleens of either 40L or AE17 tumor–bearing mice (Fig. 5A and B). In AE17 tumor–bearing mice, AMD3100 alone did not significantly reduce Treg in the lymph nodes (Fig. 5C), and AMD3100 alone or in combination with VIC-008 significantly increased the cell ratio of CD8+ T cells to Treg (P < 0.01 and P < 0.0001, respectively) compared with saline treatment (Fig. 5D). In tumors from both the 40L and AE17 models, AMD3100 applied as monotherapy significantly decreased the proportion of Treg (P < 0.05 and P < 0.01, respectively).
AMD3100 decreased PD-1 expression on CD8⁺ T cells. The proportion of PD-1-expressing cells in CD8⁺ T cells in the spleens of (A) 40L (n = 6 per group) and (B) AE17 (n = 5 per group) mice. C, The proportion of PD-1-expressing cells in CD8⁺ T cells in the lymph nodes of AE17 mice (n = 5 per group). The proportion of PD-1-expressing cells in CD8⁺ T cells in the tumors of (D) 40L (n = 6 per group) and (E) AE17 (n = 5 per group) mice. The proportion of PD-1-expressing cells in CD4⁺ T cells in the tumors of (F) 40L (n = 6 per group) and (G) AE17 (n = 5 per group) mice. H, The concentration of CXCL12 was measured by ELISA in AE17 tumors in the differently treated mice (n = 5 per group). J, CXCL12 in tumors, lymph nodes, and spleen in VIC-008-treated mice. K, Representative dot plots of PD-1 expression presented as mean fluorescence intensity (MFI) on intratumoral CD8⁺ T cells from AE17 tumors with or without AMD3100 treatment in vitro, and (K) statistical difference was analyzed (n = 5 per group). *, \( P < 0.05; **, \( P < 0.01; 
\text{and } $$$, \( P < 0.001). 
\) Data are presented as mean ± SEM; representative of two independent experiments. Statistical method: (A–I) One-way ANOVA with Dunnett multiple comparisons and (J) unpaired t test with Welch correction.
models. In these two murine mesothelioma models, AMD3100 and VIC-008 combination treatment group, the proportion of Treg was significantly decreased (P < 0.05 and P < 0.001, respectively), and the ratio of CD8+ T cells to Treg increased (P < 0.001 and P < 0.0001, respectively) compared with saline treatment in both the 40L and AE17 models. In these two murine mesothelioma models, AMD3100 reduced intratumoral Treg infiltration.

AMD3100 modulates Treg toward a T helper phenotype

We observed that AMD3100, alone or in combination with VIC-008, significantly increased the ratio of CD25+ cells to CD25+ cells within the Foxp3+ population in both 40L (Fig. 6A; P < 0.01 and P < 0.001, respectively) and AE17 tumors (Fig. 6B; P < 0.01 and P < 0.05, respectively), as well as in the lymph nodes in the AE17 model (Fig. 6C; P < 0.01 and P < 0.01, respectively). Among the Foxp3+CD25+ Treg population, significantly more cells were phenotypically IL2-CD40L+ (Fig. 6D–G) after AMD3100 monotherapy and combination therapy with VIC-008, suggesting a change to helper-like cells that have lost CD25 without the loss of Foxp3 (33), and may have lost their immunosuppressive function (34, 35). No differences in the proportion of IL2-CD40L+ cells in the Foxp3+CD25+ Treg population between AMD3100 monotherapy and combination therapy groups were seen, indicating that AMD3100 treatment may be the major driver of reprogramming Treg into helper-like cells.

The T helper phenotype is a result of TCR activation and PTEN oxidation by AMD3100

We next addressed whether AMD3100-driven Treg modulation could be initiated in isolated Treg and whether we could define its molecular basis. Cells expressing GFP-Foxp3 in CD4+ splenocytes from T-Red/Foxp3-GFP transgenic mice were sorted and treated in vitro with AMD3100. AMD3100 treatment alone did not change the proportional ratio of CD25+ cells to CD25+ cells in the Foxp3+CD4+ population (Fig. 7A and B) or the proportion of IL2-CD40L- cells in Foxp3+CD25+ Treg (Fig. 7C and D). However, in the presence of stimulation by anti-CD3/CD28 antibodies to trigger TCR activation, AMD3100 treatment significantly increased the proportional ratio of CD25+ cells to CD25+ cells in the Foxp3+CD4+ population (Fig. 7E and F; P = 0.0017) and converted more Foxp3+CD25+ Treg into IL2-CD40L- cells (Fig. 7G and H; P = 0.0015). Sharma and colleagues (33, 36) demonstrated that the inhibition of PTEN by a small-molecule inhibitor, VO-Ohpic, can cause Treg to lose CD25 without loss of Foxp3, which disrupted their immune-suppressive function. In view of this, we next examined the impact of AMD3100 on the expression of CD25 and PTEN at the single Treg level by flow cytometry. We found simultaneous downregulation of CD25 and PTEN as a result of AMD3100 treatment (Fig. 7I). This indicated that the initiation of the conversion from CD4+Foxp3+CD25+ to CD4-Foxp3+CD25+ Treg was associated with the loss of PTEN. In view of previous reports demonstrating that the loss of PTEN can be associated with oxidative inactivation (37), we examined this aspect in the context of Treg function. Using Western blot analysis, we further demonstrated that the loss of PTEN in Treg was due to oxidative inactivation after AMD3100 treatment (Fig. 7J). Together, these data demonstrated that the conversion of Treg into helper-like cells can be mediated by AMD3100 treatment upon TCR activation and PTEN oxidation.

Discussion

MSLN is overexpressed on the surface of a number of common epithelial cancers, including epithelial malignant mesothelioma, although expressed only at relatively low levels in normal mesothelial cells lining the pleura, pericardium, and peritoneum in healthy individuals (38–40). MtbHsp70 is well characterized and functions as a potent immune adjuvant (41). It stimulates monocytes and dendritic cells (DC) to produce CC-chemokines (42, 43), which attract antigen processing and presenting macrophages, DCs, and effector T and B cells (44). Antigenic peptides linked to MtbHsp70 can elicit both MHC class I–restricted CD8+ and MHC class II–restricted CD4+ T-cell responses (45–49). The fusion of the anti-MSLN scFv and MtbHsp70 takes advantage of the immune-activating action of MtbHsp70 and the tumor-targeting activity of the scFv to promote antitumor responses against the broadest profile of tumor antigens. Consistent with our previous findings in a mouse model of ovarian cancer, here we demonstrated that VIC-008 enhanced tumor-specific CD8+ T-cell–mediated cytotoxic responses and facilitated lymphocyte intratumoral infiltration in mouse models of malignant mesothelioma, resulting in significant prolongation of animal survival. However, we also found that PD-1 expression was significantly upregulated on tumor-infiltrating T cells in VIC-008–treated mice and that this was associated with high CXCL12 expression in the intratumoral microenvironment. These data indicated that the CXCL12–CXCR4 axis in the intratumoral microenvironment, itself, may modulate the PD-1/PD-L1 pathway and that the antitumor activity of tumor-infiltrating T cells could be compromised by PD-1/PD-L1 pathway activation (50). This is consistent with previous findings that CXCR4 inhibition or blockade of CXCL12 in the tumor microenvironment facilitates PD-1 blockade immunotherapy in hepatocellular carcinoma (22), pancreatic cancer (31), and colorectal cancer (32). CXCL12 and its cognate receptor, CXCR4, constitute a chemokine–receptor axis that is known to be overexpressed in the tumor microenvironment of various cancers, and activation of the CXCL12–CXCR4 axis is associated with disease progression (19, 20). In vivo and in vitro models have revealed that AMD3100, an antagonist of CXCR4, can inhibit tumor growth, metastasis, and angiogenesis by blockade of CXCL12–CXCR4 interaction and subsequent inhibition of PI3K-Akt or Ras/Raf/Erk1/2 signaling (22, 23). The CXCL12–CXCR4 axis is also known to mediate trafficking and retention of various immune cells at specific anatomic sites (51, 52). Treg are thought to mediate antitumor immune responses through selective migration to and accumulative retention at tumor sites, thereby playing an important role in the immunopathogenesis of tumors (53). For example, basal-like breast cancers behave more aggressively despite the presence of dense lymphoid infiltration due to Treg recruitment driven by hypoxia-induced upregulation of CXCR4 in Treg (54). We have reported that in an environment of elevated CXCL12 in an ovarian cancer model, blockade of the CXCL12/CXCR4 axis with AMD3100 elicited multimodal effects on tumor pathogenesis, including selective reduction of intratumoral Treg and release of antitumor effector T cells from immunosuppression, which conferred

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As I signifiant survival advantage to the ovarian tumor–bearing mice (23). In our current study, we demonstrated that AMD3100 promoted the conversion of phenotypically suppressive CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells to CD25<sup>-</sup>Foxp3<sup>+</sup>IL2<sup>+</sup>CD40L<sup>+</sup> helper-like cells (33), which may result in the loss of immunosuppressive function of intratumoral T<sub>reg</sub> (34, 35). In a similar scenario, a study on renal cell carcinoma demonstrates that CXCR4 antagonism elicited by a new peptidic antagonist, Peptide-R29, reverted the suppressive activity of T<sub>reg</sub> (50). Meiron and colleagues demonstrate, in opposition, that CXCL12 redirected the polarization of Th1 cells into IL10-producing T<sub>reg</sub> (55).

Figure 5. AMD3100 reduced tumor-infiltrating T<sub>reg</sub>. The proportion of T<sub>reg</sub> in total live splenocytes in (A) 40L (n = 6 per group) and (B) AE17 (n = 5 per group) mice. C, The proportion of T<sub>reg</sub> in total live cells in the lymph nodes of AE17 mice (n = 5 per group). D, The ratio of CD8<sup>+</sup> T cells to T<sub>reg</sub> in the lymph nodes of AE17 mice (n = 5 per group). E, The proportion of T<sub>reg</sub> in total live cells in the tumors of 40L mice (n = 6 per group). F, The ratio of CD8<sup>+</sup> T cells to T<sub>reg</sub> in the tumors of 40L mice (n = 6 per group). G, The proportion of T<sub>reg</sub> in total live cells in the tumors of AE17 mice (n = 5 per group). H, The ratio of CD8<sup>+</sup> T cells to T<sub>reg</sub> in the tumors of AE17 mice (n = 5 per group). * P < 0.05; ** P < 0.01; *** P < 0.001; and **** P < 0.0001. Data are presented as mean ± SEM, representative of two independent experiments. Statistical method: (A–H) One-way ANOVA with Dunnett multiple comparisons.
CD25\(^{-}\)Foxp3\(^{-}\)IL2\(^{+}\)CD40L\(^{+}\) helper-like cells have been demonstrated to have rapid-acting, supportive roles in priming CD8\(^{+}\) T-cell responses, but they need initial signals from activated CD8\(^{+}\) T cells to initiate the reprogramming (33, 36). Our data robustly demonstrated that AMD3100-mediated conversion of Tregs into helper-like cells was TCR activation–dependent. PTEN is a critical regulator that is capable of stabilizing the immune-suppressive function of Tregs (33, 36, 56–58). In brief, it has been shown that Tregs have high expression of PTEN, which abrogates PI3K/mTORC2-Akt pathway signaling and, thereby, maintains the expression of CD25 and Foxp3 (36, 56–58). The inhibition of PTEN results in the loss of CD25 expression, despite the maintenance of Foxp3 (33) and, thereby, the disruption of their immune-suppressive function (34, 35). In this current study, we further demonstrated that AMD3100-mediated conversion of Tregs into IL2\(^{+}\)CD40L\(^{+}\) helper-like cells was characteristic of downregulation of CD25 and associated with the loss of PTEN due to oxidative inactivation. We, thus, hypothesize that in the combinatorial treatment setting, VIC-008-activated CD8\(^{+}\) T-cell responses further facilitate AMD3100-mediated Treg reprogramming, contributing to the observed enhanced antitumor efficacy of the combination therapy.

Studies demonstrate that inhibition of CXCR4 signaling restores sensitivity to CTLA-4 and PD-1 checkpoint inhibitors (21, 22), suggesting a mechanism by which AMD3100 may modulate immune responses. Indeed, in this study, we found that AMD3100 was associated with suppression of PD-1 expression on CD8\(^{+}\) T cells, consistent with the abrogation of...
Figure 7.
AMD3100-driven T<sup>reg</sup> reprogramming required TCR activation. A, Representative dot plots of the Foxp3<sup>+</sup>CD25<sup>−</sup> and Foxp3<sup>+</sup>CD25<sup>+</sup> population with or without AMD3100 treatment under no anti-CD3/CD28 stimulation and (B) statistical analysis of the dataset (n = 4 per group). C, Representative density plots of IL2<sup>+</sup>CD40L<sup>+</sup> cells in the Foxp3<sup>+</sup>CD25<sup>−</sup> T<sub>reg</sub> population with or without AMD3100 treatment under no anti-CD3/CD28 stimulation and (D) statistical analysis of the dataset (n = 4 per group). E, Representative dot plots of the Foxp3<sup>+</sup>CD25<sup>−</sup> and Foxp3<sup>+</sup>CD25<sup>+</sup> population with or without AMD3100 treatment under anti-CD3/CD28 stimulation and (F) statistical analysis of the dataset (n = 4 per group). G, Representative density plots of IL2<sup>+</sup>CD40L<sup>+</sup> cells in the Foxp3<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> population with or without AMD3100 treatment under anti-CD3/CD28 stimulation and (H) statistical analysis of the dataset (n = 4 per group). I, Representative dot plots of CD25 and PTEN on CD4<sup>+</sup>Foxp3<sup>+</sup> T cells with and without AMD3100 treatment in vitro under anti-CD3/CD28 stimulation. J, Western blot analysis of PTEN in CD4<sup>+</sup>Foxp3<sup>+</sup> T cells with and without AMD3100 treatment in vitro. Statistical difference was analyzed using unpaired t test with Welch correction. Data are presented as mean ± SEM; representative of two independent experiments.
PD-1–mediated immunosuppression. Our findings demonstrated that the augmentation of tumor-specific CD8+ T-cell responses induced by VIC-008, together with the abrogation of immune suppression mediated by AMD3100, both through inhibition of PD-1 expression on intratumoral CD8+ T cells and through conversion of suppressive Treg to helper-like cells, conferred significant benefits on tumor control and animal survival in malignant mesothelioma. A clear next step would be to investigate how AMD3100 affects other checkpoint molecules, including LAG-3, TIM-3, and TIGIT.

Taken together, these data revealed the therapeutic potential of AMD3100, highlighting its potential to act as a potent immune modulator that can complement the activity of targeted cancer immunotherapies, such as DC- or T-cell–based vaccines. These data also highlighted that vaccine-based cancer immunotherapy may be further improved if Treg are quantitatively reduced or functionally impaired. In this way, our study may offer a therapeutic approach for significantly prolonging the survival of patients with malignant mesothelioma and expand the potential clinical efficacy of both novel and established DC- or T-cell–based vaccines and immunotherapies for this difficult-to-treat disease.

Disclosure of Potential Conflicts of Interest

J.A. Gelfand is a consultant/advisory board member for Supportive Therapeutics and Henry Schein, Inc. T.A. Brauns has ownership interest (including patents) in AperiSys, Inc. M.C. Poznansky is a scientific consultant at AperiSys, Inc. and has ownership interest (including patents) and founder’s equity. No potential conflicts of interest were disclosed by the other authors.

References


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Intratumoral Immunosuppression Immune-Activating VIC-008 in Mesothelioma by Modulating Intratumoral Immunosuppression

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