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CXCR4 inhibition modulates the tumor microenvironment and retards the growth of B16-OVA melanoma and Renca tumors

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Abstract

Objectives and Methods: To determine if blockade of the chemokine receptor CXCR4 might alter the tumor microenvironment and inhibit tumor growth, we tested the efficacy of the CXCR4 antagonist X4-136 as a single agent and in combination with various immune checkpoint inhibitors in the syngeneic murine melanoma model B16-OVA. We also tested its activity alone and in combination with axitinib in the renal cancer model Renca.

Results: We found that X4-136 exhibited potent single agent antitumor activity in the B16-OVA model that was additive to that of an anti-PDL1 antibody. The antitumor activities were associated with a reduction in the number of immunosuppressive regulatory T-cells and myeloid-derived suppressor cells and an increase in the number of tumor-specific CD8⁺/perforin⁺ cells in the tumor-microenvironment. Apart from these immune effects, X4-136 alone and in combination with checkpoint inhibitors inhibited the Akt/FOXO-3a cell survival pathway *in vitro* and *in vivo*, suggesting that it might have antitumor activity independent of its effects on immune cell trafficking. Similar effects on tumor growth and CTL infiltration were observed in the Renca model.

Conclusions: These studies show that the effects of CXCR4 blockade on immune cell trafficking might serve as a useful adjunct to immune checkpoint inhibitors and other therapies in the treatment of cancer.

Keywords

melanoma; renal cell carcinoma (RCC); CXCR4 inhibition; checkpoint inhibitors; immunotherapy; myeloid-derived suppressor cells (MDSC); regulatory T cells

Introduction

Melanoma is an aggressive, potentially lethal cancer [1, 2] for which several new therapeutic modalities have recently emerged. Targeted therapies directed against the common BRAFV600E mutation and MEK have been shown to increase median progression-free survival and are now FDA-approved for the treatment of patients with melanoma [3-6].

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Immune checkpoint inhibitors which incapacitate an important mechanism of tumor-mediated immune evasion have also been approved for these patients [7]. Ipilimumab, an anti-CTLA-4 monoclonal antibody, was the first of these agents shown to prolong the overall survival in metastatic melanoma [8]. Another checkpoint crucial as a tumor escape mechanism is PD-1. Several inhibitors against PD-1 or its ligand PD-L1 have been developed and have been shown to induce durable response rates of 30–40% in melanoma patients [9, 10]. However, despite these successes, the majority of the patients obtain only partial remissions [11], indicating a need for additional or alternative approaches.

CXCR4 is a member of a family of structurally related G-protein-coupled receptors [12] expressed by numerous cell types including T lymphocytes, monocytes, neutrophils, and endothelial cells [13]. CXCR4 exerts its biological effect upon binding its ligand chemokine ligand 12 (CXCL12) or stromal-derived-factor (SDF-1), which is expressed by stromal cells including fibroblasts and endothelial cells. CXCL12 binding to CXCR4 triggers multiple downstream signal transduction pathways such as the phosphorylation of ERK1/2, p38, SAP/JNK, AKT, mTOR and the Bruton tyrosine kinase (BTK). Activation of these pathways leads to an alteration of gene expression which is critical in cell proliferation, cell skeleton rearrangement, angiogenesis, cell migration, chemotaxis, and cell survival [14–18]. Several lines of evidence suggest that the chemokine receptor CXCR4 is over-expressed by a variety of solid tumors and hematopoietic malignancies, including ovarian, prostate, esophageal, and renal cell carcinomas as well as melanoma and neuroblastoma [19]. Studies indicate that CXCR4 is upregulated in melanoma at the mRNA and protein levels and patients with CXCR4-positive melanoma have a 3.1-fold higher risk of death due to disease compared to patients with CXCR4-negative tumors [14]. Thus, CXCR4 expression serves as a strong independent prognostic factor.

CXCR4 plays a critical role in cancer cell survival and metastasis [20], possibly by activating pro-survival signals that render cancer cells resistant to immune attack [13]. CXCR4 is essential for the metastatic spread of cancer cells to organs where CXCL12 is expressed and thereby allows tumor cells to access cellular niches such as bone marrow, liver, lungs and lymph nodes that support tumor-cell survival and growth [21, 22]. Targeted metastasis to the marrow or other sites of high CXCL12 expression involves CXCR4 activation on circulating tumor cells that “hijack” the CXCR4-CXCL12 axis for homing to various microenvironments that normally are restricted to hematopoietic progenitor cells (HPCs). Stromal-derived CXCL12 can stimulate survival and growth of neoplastic cells in a paracrine fashion by binding to CXCR4 on the tumor cells.

T cell-mediated tumor immunity depends on the migration and co-localization of cytotoxic T lymphocytes with tumor cells, a process regulated by chemokines and adhesion molecules. Normal tissues such as bone marrow and dysplastic tissues producing abundant CXCL12 are rarely infiltrated by significant number of T-cells. It has been shown that high levels of CXCL12 actually induce chemorepulsion of tumor-specific CTL and thereby favors tumor growth by limiting tumor infiltration by effector T cells [13]. Multiple studies have shown that inhibition of CXCR4 via antibodies or peptide antagonists reduces metastasis and/or tumor growth. CXCR4 antagonists have also been evaluated for inhibition of cross talk between tumor and stromal cells and for the mobilization of cancer cells from the protective

microenvironment of solid tumors, making them more sensitive to conventional chemotherapy, radiotherapy, or anti-angiogenic therapy [22-26].

In the previous studies from our lab, we have shown that the combination of a CXCR4 antagonist with the anti-angiogenic agent axitinib reduced myeloid-derived suppressor cell tumor infiltration and had synergistic anti-tumor effects in RCC xenografts [27]. In order to further explore the mechanism of action of CXCR4 inhibitors in an immune-proficient background, we investigated the activity of the CXCR4 antagonist X4-136 in syngeneic mouse models. In the present study, we have tested the efficacy of X4-136 as single agent and in combination with checkpoint inhibitors in melanoma and with axitinib in an RCC model.

Materials and Methods

Cell lines and reagents

B16 melanoma cells (H2^b) stably expressing chicken ovalbumin (B16-OVA) were provided by Dr. Ben Izar, Dana Farber Cancer Institute, Boston, MA. These cells were found to express low levels of PD-L1. 5.6% of B16-ova cells were found to be PD-L1 positive (Supplementary Fig. 1). The murine RCC cell line Renca DM (Renca-2159-84H) was obtained from Dr. Gordon Freeman, Dana Farber Cancer Institute. These cells were transfected with a HIF-2 α construct with two mutations which prevent proteasomal degradation. The cell lines were maintained in RPMI-1640 (Lonza) containing 10% fetal bovine serum (USA Scientific), 2 mM glutamine, and 50 μ g/ml gentamycin at 37°C in 5 percent CO₂. The axitinib was purchased from LC Labs (Woburn, MA). Anti-PD-1, anti-PD-L1, and anti-CTLA-4 antibodies were purchased from Bio-X-Cell and X4-136 was provided by X4 Pharmaceuticals, Cambridge, MA.

Mice

All animal studies were conducted according to an (IACUC)-approved protocol at the BIDMC. Six to eight week old C57BL/6 and Balb/c female mice (Charles River Labs) were implanted subcutaneously with 1.0×10^5 B16-OVA or Renca DM cells, respectively, in the right flank. For the B16-OVA model, treatment was started after tumors attained a diameter of 3 mm. Mice were randomly grouped with 6 mice per group and treated for 16 days with vehicle (5-days on/ 2-days off), X4-136 (100 mg/kg by gavage, 5-days on/2-day off), anti-PD-L1 (100 μ g/mouse IP every alternate day), anti-PD-1 (100 μ g/mouse IP every alternate day), a combination of anti-PD-L1+X4-136, or a combination of anti-PD-1+X4-136. In a separate experiment, mice were treated for 16 days on the same schedule with vehicle, X4-136 (100 mg/kg), anti-PD-L1(100 μ g/mouse IP every alternate day) + anti-CTLA-4 (100 μ g/mouse IP every fourth day), or anti-PD-L1 + anti-CTLA-4 + X4-136.

For the Renca model, treatment was started when the tumors attained a diameter of 6 mm. Mice were randomized into 4 groups and treated for 8 days with vehicle, X4-136 (100 mg/kg, 5-days on/2-day off), axitinib (30 mg/kg by gavage, 5-days on/2-day off), or axitinib + X4-136. All treatments in both the models were well tolerated. Tumors were measured bidimensionally daily and tumor volume was calculated using formula $V = (\text{smallest}$

diameter)² X the largest diameter /2. After the mice were sacrificed, tumors were excised and the tissue was frozen in liquid N₂ for western blot analysis or quantitative real time PCR. A portion of the excised tumors was used to prepare single cell suspensions for flow cytometry.

RNA preparation, PCR Array, and Real Time qPCR

Total RNA was isolated from tumor tissue using Purelink RNA Minikits (Ambion, Life Technologies) according to the manufacturer's instructions. The quality and quantity of RNA samples were measured using a spectrophotometer (Amersham Biosciences, Little Chalfont, UK). An A260/A280 ratio of 1.8–2.0 for RNA was considered suitable for real time qPCR. cDNA was prepared using the Reverse Transcriptase Mix (Qiagen Ltd.). Quantitative PCR was carried out in accordance with the RT² Profiler PCR array instructions. Cancer Inflammation and Immunity Crosstalk PCR Arrays (Qiagen) were used to analyze the expression of 84 immune-related genes, a subset of which (CTLA-4, FOXP3, granzyme B, and IDO1) were selected for further study by real time quantitative PCR using the Taqman probes for specific genes (Applied Biosystems) according to manufacturer's instructions. The relative mRNA values were calculated by double delta method and normalized to the level of housekeeping gene GusB.

Western blots

Tumor tissues and tumor cells were lysed in RIPA buffer (Cell Signaling) supplemented with sodium fluoride (10 μ M, Fisher Scientific, Hampton, NH) and phenylmethylsulfonyl fluoride (100 μ g/ml, Sigma-Aldrich, St Louis, MO). For some in-vitro experiments, cells were maintained in hypoxia (1% O₂) in hypoxic incubator (Biospherix, Model E702). Lysates were fractionated in 8–16% gradient SDS-polyacrylamide gels and the separated proteins were transferred to nitrocellulose membranes. The blots were probed for the proteins of interest with specific antibodies followed by a secondary antibody-horse radish peroxidase conjugate and then incubated with SuperSignal chemiluminescence substrate (Pierce, Rochford, IL). The blots were then exposed to Kodak X-Omat Blue XB-1 film. The p-Akt, Akt, p-FOXO-3a, FOXO-3a, cyclin D1, IDO1 and β -Actin antibodies were purchased from Cell Signaling Technologies (Beverly, MA) and the HRP-conjugated anti-rabbit and anti-mouse antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of single cell suspensions and flow cytometry

Single cell suspensions from tumor tissue and lymph nodes were prepared and analyzed for different immune cell populations by flow cytometry. Briefly, the tissues were minced into small pieces using scissors and incubated with 200U/ml collagenase (Thermo Fisher Scientific, Waltham, MA) and 100U/ml DNase I (Sigma Aldrich, St. Louis, MO) in RPMI for 30 min at 37°C. Single cell suspensions thus obtained were stained with the following directly conjugated anti-mouse antibodies (Biolegend, San Diego, CA): Alexa Flour 488-anti-CD3, Alexa Flour 488-anti-CD8a, APC-anti-perforin. For MDSC, cells were stained with Alexa Flour 647-anti-Gr-1 and Alexa Fluor 488-anti-CD11b. For Tregs, the True Nuclear OneStep Staining Mouse Treg Flow kit (FOXP3 Alexa Flour 488/CD25 PE/CD4PerCP) was used as per manufacturer's instructions (Biolegend). In a separate experiment, a PE-conjugated H-2K^b/ SIINFEKL tetramer (MBL International Corporation,

Woburn, MA) was used to quantitate OVA-specific CD8⁺ T cells. Tetramer staining was carried out in accordance with the manufacturer's suggested protocol. Flow cytometric analysis was performed using Gallios software and a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Statistical analysis

Graph Pad Prism was used for statistical analysis and the differences between groups was calculated using One-way ANOVA and Student's t-test. p-values <0.05 were considered statistically significant.

Results:

CXCR4 inhibition with X4-136 suppresses the growth of B16-OVA tumors alone and in combination with immune checkpoint inhibition.

To assess the activity of X4-136 in a melanoma model, mice bearing B16-OVA tumors were treated with X4-136 alone or in combination with various immune checkpoint inhibitors. We found that X4-136 alone displayed significant tumor growth retardation (Fig. 1A, $p < 0.001$ for X4-136 vs. control) that was better than that achieved with the checkpoint inhibitors anti-PD-1 (Fig. 1A, $p < 0.01$ for X4-136 vs. anti-PD-1, $p > 0.05$ for anti-PD-1 vs. control) or anti-PD-L1 (Fig. 1B, $p < 0.05$ for anti-PD-L1 vs. X4-136 and control). When X4-136 was administered in combination with these checkpoint inhibitors, synergistic anti-tumor activity was observed with anti-PD-L1 (Fig. 1B, $p < 0.001$ for the combination vs. X4-136 alone) but not with anti-PD-1 (Fig. 1A) or the combination of anti-PD-L1 and anti-CTLA-4 (Fig. 1C). In fact, single agent X4-136 appeared to have at least as much antitumor activity as the combinations of anti-CTLA-4 + anti-PD-L1 or all three agents.

CXCR4 inhibition affects T-cell infiltration and activity alone and in combination with immune checkpoint inhibition.

To study the effects of CXCR4 inhibition on circulating leukocytes, we quantified WBC levels in the peripheral blood in mice undergoing treatment with X4-136 (Supplementary Fig. 2). We found that 2 hours after a dose of X4-136, peripheral WBC counts increased by two-fold ($p < 0.001$ for X4-136 at 2h vs. 0h). There was no change in WBC level in the vehicle-treated group.

Next, we analyzed the effect of treatment on various effector T-cell populations in the tumor microenvironment. The percentage of CD3⁺ cells in the tumor microenvironment in vehicle-treated mice was found to be 2.28 % and treatment with X4-136 led to a 2.7-fold increase ($p < 0.001$) in total CD3⁺ cell infiltration over the vehicle-treated group (Fig. 2A). Treatment with either anti-PD-1 or anti-PD-L1 alone did not significantly alter the extent of the infiltration. However, the PD-L1 antibody augmented the effect of X4-136 as the combination resulted in a 5.8-fold increase in infiltrating CD3⁺ cells ($p < 0.001$ and $p < 0.01$ for the combination vs. control and X4-136, respectively). In a separate experiment in which X4-136 alone was shown to induce a 4.7-fold increase in CD3⁺ cell accumulation, the combination of X4-136 with both the anti-PD-L1 and anti-CTLA-4 antibodies led to a 10-fold increase in CD3⁺ cell infiltration (Supplementary Fig 3A, $p < 0.001$ for the 3-drug

combination vs. control and X4–136). Similar trends were seen with CD8⁺ cell infiltration (Fig. 2B and Supplementary Fig. 3B). As shown in Fig. 2B, levels of total CD8⁺ cells increased 4.2-fold (from 2.6% to 11%) on treatment with X4–136 ($p<0.05$ for X4–136 vs. vehicle) and co-treatment with X4–136 and anti-PD-L1 led to superior level of such TIL in comparison to either monotherapy ($p<0.05$ and <0.001 for the combination vs. X4–136 and PD-L1, respectively). Activated CD8⁺ T cells (i.e. those co-expressing perforin) accounted for 0.07% of the cells in control tumors. CXCR4 inhibition alone led to a robust 18.5-fold increase in these cells (Fig. 2C, $p<0.001$). The combination of X4–136 with anti-PD-1, anti-PD-L1, or anti-CTLA-4 + anti-PD-L1 led to further increases of up to 41-, 91- and 38-fold, respectively (Fig. 2C and Supplementary Fig. 3C, $p<0.001$ for all 3 combinations vs control; $p<0.05$, $p<0.001$, and $p<0.05$ for the combinations vs. single agent X4–136, respectively). OVA peptide-specific CD8⁺ TIL frequencies were analyzed using a specific H-2Kb/OVA peptide tetramer probe (Fig. 2D and Supplementary Fig. 3D). While tumors from the vehicle-treated mice had 0.43% CD8⁺/tetramer⁺ cells, treatment with X4–136, anti-PD-1, or anti-PD-L1 resulted in a 3-, 4-, and 7- fold increase in these cells respectively ($p<0.01$ vs. control). Furthermore, as shown in figure 2D, the combination X4–136 and anti-PD-L1 induced a synergistic (11.9-fold) increase in their number ($p<0.01$ for the combination vs. X4–136 and $p<0.01$ for the combination vs. anti-PD-L1). Similar results were obtained with the combination of X4–136 with anti-CTLA-4 + anti-PDL1 in a separate experiment (Supplementary Fig. 3D). In this study, tumors from vehicle-treated mice had 0.69% CD8⁺/tetramer⁺ cells. Those from mice treated with X4–136 or the combination of anti-CTLA-4 + anti-PD-L1 had a 9.1- and 4.7- fold increase in these cells ($p<0.001$ and $p<0.01$ for X4–136 and the combination of anti-CTLA-4 + anti-PD-L1 vs. control, respectively). The addition of X4–136 to the combination of anti-CTLA-4 + anti-PD-L1 led to 29.4-fold increase in CD8⁺/tetramer⁺ cells over control ($p<0.001$ for the triple combination vs control). This triple combination was superior to both X4–136 alone and the combination of anti-CTLA-4 + anti-PD-L1 ($p<0.001$ for the triple combination vs. X4–136 alone and vs. the combination of anti-CTLA-4 + anti-PD-L1).

To determine if CXCR4 inhibition had an effect on the number of tumor antigen-specific CD8⁺ T cells present within lymph nodes, nodes were disaggregated and analyzed by flow cytometry. While lymph nodes from vehicle-treated mice had 6% CD8⁺/tetramer⁺ cells, CXCR4 inhibition led to more than 2.6-fold increase in OVA peptide-specific CD8⁺ T cells in comparison to treatment with vehicle (Supplementary Fig. 4, $p<0.05$ for X4–136-treated vs. control). No significant change was observed with anti-PD-1 or anti-PD-L1 treatment. However, when X4–136 was combined with anti-PD-1 or anti-PD-L1, the percentage of tetramer⁺ cells increased 3.5-fold ($p<0.01$ for the combination vs control; $p<0.05$ combination vs. X4–136 alone), suggesting an additive effect.

CXCR4 inhibition reduces immune-regulatory cell populations in the melanoma microenvironment.

To assess the effect of CXCR4 inhibition on the trafficking of immunosuppressive leukocytes, we examined the impact of single agent X4–136 and various immune checkpoint inhibitory antibodies on myeloid-derived suppressor cells (MDSC) and Treg cells in melanoma-bearing mice. The levels of MDSC and Tregs in the tumors of vehicle-treated

mice were 4.7% and 1.7%, respectively. Figures 2E and 2F show that treatment with X4-136, PD-1 or PD-L1 reduced MDSCs by 72, 55, and 85%, respectively ($p < 0.01$ for each modality vs. control) and Tregs by 68, 61, and 84 % respectively ($p < 0.001$ for each modality vs. control) compared to treatment with vehicle. Combination of X4-136 with anti-PD-L1 led to a further reduction in both MDSC and Treg populations by 89% and 86% in comparison to the vehicle-treated group ($p < 0.001$ for the combination vs. control, $p < 0.05$ for the combination vs. X4-136 and PD-1 individually). Similar trends were observed when X4-136 was combined with anti-CTLA-4 + anti-PD-L1 (Supplementary Fig. 3E and F). X4-136 and the combination of anti-CTLA-4 + anti-PD-L1 led to 25% and 30% reductions in MDSC levels respectively, ($p < 0.05$ for X4-136 vs. control, $p < 0.05$ for the antibody combination vs control). The triple combination decreased MDSC levels by 52% ($p < 0.05$ for the triple combination vs. X4-136 and vs. the dual anti-CTLA-4 + anti-PD-L1 antibodies). While X4-136 and the combination of anti-CTLA-4 + anti-PD-L1 led to 35 and 30% reductions in Tregs, respectively ($p < 0.05$ treatment vs. control), the combination therapy led to a decrease by 55% in these cells ($p < 0.01$ triple combination vs. control and $p < 0.05$ for the triple combination vs. X4-136 and the combination of the two antibodies).

To further define the effects of treatment on leukocyte trafficking, a PCR- based array analysis for lineage-specific immune regulatory genes was performed. Several of these genes were modulated with CXCR4 inhibition and a select group was further studied by RT-PCR. Figure 3A shows that CXCR4 inhibition led to a decrease in the mRNA level of the checkpoint inhibitor CTLA-4 by 79% ($p < 0.001$ X4-136 vs. vehicle-treated group). With anti-PD-L1 treatment, no significant change was observed. However, co-treatment of X4-136 with anti-PD-L1 led to a further reduction in the level of CTLA-4 ($p < 0.05$ vs X4-136-treated group). Figure 3B shows that granzyme B mRNA levels were upregulated 6.4-fold in the X4-136 alone arm ($p < 0.001$ X4-136-treated vs. control) and 3.3-fold in the PD-L1 arm ($p < 0.01$ treatment vs. control). In the combination arm, a further increase of up to 32.5-fold was observed ($p < 0.001$ combination vs. control and vs. single agents). As shown in Figure 3C, the levels of mRNA for Foxp3, a marker of Tregs, was reduced by 70 and 60 % by single agents X4-136 and anti-PD-L1 ($p < 0.01$), respectively, and the combination of these treatment modalities led to a further decrease of 88% ($p < 0.05$ combination vs single agents).

CXCR-4 inhibition also led to drastic decrease (by 90% compared to control) in the level of mRNA for IDO1, the products of which (kynurenines) are known to inhibit the activities of CD8⁺ cells ($p < 0.001$) (Fig 3D). When anti-PD-L1 was administered along with X4-136, a further decrease (by 96%) in IDO mRNA was observed ($p < 0.01$ combination vs. X4-136-treated group). The reduction in IDO-1 expression at the mRNA level was confirmed at the protein level by immunoblotting. X4-136 alone and in combination with anti-PD-L1 almost completely inhibited the expression of IDO1 at the protein level (Fig 3E).

X4-136 alone and in combination with immune checkpoint inhibitors blocks activation of Akt/FOXO-3a pathway in melanoma and leads to suppression of cyclin D1 expression.

Next we analyzed the effect of CXCR4 inhibition on downstream effectors of the CXCR4 pathway (Figs 4A and B). Treatment with X4-136 alone led to a decrease in phosphorylated Akt and in the phosphorylation of its substrate FOXO-3a. Dephosphorylation of FOXO-3a

prevents its proteasomal degradation, leading to the accumulation of FOXO-3a protein. When X4-136 was combined with an anti-PD1 (panel A) or anti-PDL1 (panel B) antibody, a further decrease in the levels of p-Akt and p-FOXO-3a was noted along with an increase in the level of unphosphorylated FOXO3a. We also observed a decrease in the level of cyclin D1 in tumors from mice treated with the anti-PDL1 combination therapy.

Further, we analyzed the key signaling molecules from the pathway in B16-OVA cells *invitro* under normoxic and hypoxic conditions (Fig 4C). The in-vitro results corroborate the *in vivo* findings, we observed a reduction in the levels of p-Akt and p-FOXO-3a on treatment with X4-136 under hypoxic condition. We also observed increase in the level of unphosphorylated FOXO-3a and decrease in cyclin D1 expression when B16-OVA cells were treated with 5 and 10uM of X4-136 in hypoxic conditions.

CXCR4 inhibition by X4-136 also inhibits the growth of renal cell carcinoma in Renca-DM syngeneic model.

In order to explore the anti-tumor activity of CXCR4 inhibition in other syngeneic cancer models, we assessed the activity of X4-136 in the Renca model of renal cell carcinoma. As the current murine cell lines of RCC do not share the same genetic alterations as the human disease, we used a modified Renca cell line (Renca-DM) that expresses a stable, doubly mutated form of HIF-2 α . In these studies, X4-136 was tested as single agent and in combination with the anti-VEGFR agent axitinib, an FDA-approved standard treatment for RCC. Fig. 5A shows that both X4-136 and axitinib led to a significant decrease in tumor growth as compared to vehicle-treated mice after 8 days of treatment ($p < 0.001$ drugs vs. control). Synergistic anti-tumor activity was seen when the two agents were administered together ($p < 0.001$ combination vs. either monotherapy).

We also assessed the effects of treatment on the different immune cell populations in the tumor microenvironment by flow cytometry. We observed that after 8 days of treatment, the control group had 1.9% CD3⁺ cells. The single agents X4-136 and axitinib induced a slight increase (1.4-fold) in CD3⁺ cells ($p < 0.05$ drugs vs. control) but the combination therapy led to more than a two-fold increase in infiltration by CD3⁺ cells (Fig 5B, $p < 0.01$ combination vs. control). While total CD8⁺ cell levels in the control group was 1.1 % and not significantly altered by any of the treatment regimens (Fig. 5C), the levels of active CD8⁺/perforin⁺ cells were significantly increased by both single agents ($p < 0.05$ drugs vs. vehicle) and the infiltration was further enhanced (3.4-fold) by combination therapy ($p < 0.001$ combination vs. control; $p < 0.05$ combination vs. single agents) (Fig. 5D).

In the control group, 3.15% of tumor cells were MDSCs. Axitinib treatment led to a 2.5-fold increase in MDSC levels ($p < 0.05$ vs. control). Significant changes were not observed in the levels of MDSC in tumors with X4-136 monotherapy, although the drug did prevent the increase in MDSC otherwise induced by axitinib treatment ($p < 0.05$ combination vs. axitinib). The level of Tregs in the tumor microenvironment was 5% in vehicle-treated mice and unaffected by axitinib. However, it was reduced by half by X4-136 treatment ($p < 0.01$) and a further reduction to 25% of the level seen in vehicle-treated mice was seen when X4-136 was administered along with axitinib ($p < 0.001$ combination vs control and axitinib;

$p < 0.05$ for the combination vs. X4–136). We were unable to obtain data at time points later than Day 8 due to the tendency of the Renca tumors to ulcerate through the skin.

Discussion

This study demonstrates that the CXCR4 inhibitor X4–136 retards the growth of B16-OVA and Renca tumors. This antitumor effect was associated with an increase in the number of activated antigen-specific CD8⁺ T cells and a corresponding decrease in the number of Tregs and MDSC infiltrating the tumors. CXCR4 is known to be expressed on numerous cell types including T-lymphocytes, monocytes, neutrophils, and endothelial cells [28]. High expression of the CXCR4 ligand CXCL12 has also been shown to repel tumor-specific effector T cells and to recruit suppressive cell populations at tumor sites, including interleukin-10–producing dendritic cells, regulatory T cells (Treg), and myeloid-derived suppressor cells [29]. These observations suggest that the antitumor activity of X4–136 we have observed in the B16-OVA and Renca models may be have been due to treatment-induced changes in the migration of cells that constitute the tumor microenvironment.

Vianello *et al* have demonstrated that while CXCL12 at low concentrations (<10nM) acts as T cell chemoattractant, higher concentrations of the chemokine can repel T-cells *in vitro* and *in vivo* via a CXCR4 receptor–mediated mechanism. This process is termed chemorepulsion or fugetaxis [13]. Their results demonstrated that the expression of high levels of CXCL12 by a genetically modified B16 melanoma induces tumor-specific CTL chemorepulsion and thereby favors tumor growth by limiting the influx of effector T cells. They hypothesized that CXCL12 exerts its chemorepellant action on T cells primarily within the tumor microenvironment because of the chemokine's high affinity binding to extracellular matrix proteins [30,31]. Their study showed that the reduced T cell infiltration into tumors expressing high levels of CXCR4 could be reversed by CXCR4 blockade. Our results showing increased recruitment of activated T cells and diminished accumulation of MDSC and Tregs in B16-OVA grafts from mice treated with X4–136 support this hypothesis.

X4–136 also reduces the extent to which tumors are infiltrated by immunosuppressive cell populations including Tregs and MDSC. It is possible that the decrease in Tregs in the tumors of mice treated with X4–136 could be due to the particularly abundant expression of CXCR4 on these cells [29, 32] and the possibility that their trafficking is regulated predominantly by the SDF-1/CXCR4 axis whereas that of other leukocytes may be responsive to numerous chemokines.

As an adjunct to our flow cytometric analyses, we have assayed the expression of immune cell lineage-specific genes in tumor tissue and have observed that CXCR4 inhibition modulates the expression of several such genes, confirming our flow cytometric data. A drastic reduction in the expression of the immune checkpoint CTLA-4 was observed on CXCR4 inhibition. The expression of CTLA-4 is associated with negative effects on T cell activation and, as mentioned previously, CTLA-4 is abundantly expressed on CD4⁺CD25⁺ regulatory T-cells. Its expression is not simply a marker for Tregs but has been implicated in their function as well [33]. CTLA-4 is also required for TGF- β to induce FOXP3 in CD4⁺CD25⁺ regulatory cells [34]. The suppressive effect of CXCR4 inhibition on CTLA-4

expression could therefore account in part for the observed reduction in the levels of FOXP3 mRNA in tumors resulting from treatment with X4–136, independent of the effect of CXCR4 blockade on Treg migration into tumor infiltrates.

The molecular mechanism by which CXCR4 signaling influences FOXP3 expression is not entirely clear [35]. One possibility is that it affects the methylation status of the Treg-specific demethylated region (TSDR) in the *FOXP3* gene, a region whose demethylation leads to stable FOXP3 expression in Tregs [36]. Previous studies have shown reduced demethylation at this site when RCC-derived Tregs were treated with CXCR4 antagonists [32]. It is also possible that CXCR4 modulation affects FOXP3 expression through a mechanism that involves changes in the acetylation status of *FOXP3* gene promoter [35].

We also observed an increase in granzyme B mRNA with X4–136 treatment that corroborates the flow data showing an increase in the number of activated (i.e. perforin-expressing) CD8⁺ T cells within the tumor infiltrates of treated melanomas.. Finally, we have observed a complete suppression of indoleamine dioxygenase (IDO-1) expression with CXCR4 inhibition. IDO catalyzes the metabolism of L-tryptophan, resulting in the production of kynurenines, which have been implicated as a major mechanism of immunosuppression in tumors [37]. IDO inhibits the activation of effector T-cells through depletion of tryptophan and promotes differentiation and activation of Foxp3⁺ Tregs through production of tryptophan metabolites (kynurenines) [38]. Holmgaard *et al* demonstrated that IDO orchestrates local and systemic immunosuppression through recruitment and activation of MDSCs, through a mechanism dependent on Tregs. They demonstrated that IDO expression in human melanoma tumors is strongly associated with MDSC infiltration and treatment with selective IDO inhibitor decreased number of infiltrating MDSCs and Tregs, abolishing their suppressive functions [37]. Thus, excluding IDO-expressing cells from the tumor microenvironment would be expected to lead to both activation of effector T cells and suppression of MDSC. It is therefore conceivable that the suppression of IDO expression (or exclusion of IDO-expressing cells from the tumor microenvironment) mediates some of the effects of CXCR4 inhibition on leukocyte trafficking observed in our studies with X4–136.

CXCR4 is known to activate a number of signaling pathways including Erk1/2, p38, SAPK/JNK, Akt, mTOR and Bruton tyrosine kinase (BTK) that regulate intracellular calcium flux, chemotaxis, transcription, cell survival, and migration in immune cells and tumor cells [14, 39]. Akt serves as a central node in CXCR4/SDF-1 signaling cascade and modulates cell proliferation and motility [40]. Our data shows that treatment with X4–136 alone led to a decrease in the phosphorylation of Akt and that of its substrate FOXO-3a, resulting in the intracellular accumulation of FOXO-3a protein, which would be expected to augment the expression of several genes associated with diminished cellular survival. The modulation of Akt/FOXO-3a pathway in melanoma cells under hypoxic conditions suggests that the X4–136 may have intrinsic antitumor effects independent of its effects on leukocyte trafficking. The remarkable single agent activity of X4–136 in suppressing the growth of melanoma and RCC tumors raises the possibility that the compound may have a direct effect on tumor cells independent of its immunologic effects.

We also tested the efficacy of X4–136 in an RCC syngeneic model as a single agent and in combination with the anti-VEGFR agent axitinib. X4–136 treatment led to significant suppression of tumor growth in the Renca model with synergistic effects obtained with axitinib co-treatment. X4–136 treatment augmented the influx of CD8⁺/perforin⁺ cells and reduced that of Tregs, similar to its effects in the B16-OVA model but had little effect on MDSC infiltration. It should be borne in mind, however, that subcutaneous Renca implants have a tendency to ulcerate prematurely and that these studies were therefore done with Day 8 tumors. It is therefore possible the effects of treatment would have been more pronounced if it were not for the abbreviated treatment course we were compelled to utilize in our studies with this model.

Cancer immunotherapy and in particular monoclonal antibodies blocking the inhibitory programmed cell death 1 pathway (PD-1/PD-L1) have made a significant impact on the treatment of cancer, not only in melanoma and RCC, but also in tumors previously not considered immunogenic [41–44]. Despite clinical successes, their effects are not curative or even durable in most patients [23, 45]. Therefore, the ideal therapeutic approach should be aimed at simultaneous activation of T-cells that specifically target tumor cells and inhibition of tumor influx of immunosuppressive cells. The remarkable single agent activity of CXCR4 antagonist X4–136 observed in our study and the additive effects observed with anti-PD-L1 and axitinib provide evidence that CXCR4 blockade might serve both as a monotherapy and as a useful adjunct to immune checkpoint inhibitors in the treatment of melanoma and RCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1: Effect of X4-136 and immune checkpoint inhibitor antibodies on the growth of syngeneic B16-OVA melanomas.

Mice were treated with X4-136, anti-PD-1, anti-PD-L1 or anti-CTLA-4 + anti-PD-L1 individually or in combination as depicted. Tumor growth was plotted as mean $\pm$ SEM for $n=5-6$ for each treatment group.

Figure 2: Increased infiltration of cytotoxic T-cells and reduction of immunosuppressive cell populations in the tumor micro-environment.

Melanomas were harvested after 16 days of treatment and digested. Single cell suspensions were analyzed by flow cytometry for (A) CD3⁺ cells, (B) CD8⁺ cells, (C) CD8⁺ perforin⁺ lymphocytes, (D) OVA-specific (i.e. those binding the PE-conjugated H-2K^b/SIINFEKL tetramer) CD8⁺ lymphocytes, (E) myeloid-derived suppressor cells (MDSC), and (F) regulatory T-cells (Tregs). The results are shown as fold change in number of cells relative to control and expressed as mean $\pm$ SEM for n=5-6.

Figure 3: X4-136 alone and in combination with immune checkpoint inhibitors leads to suppression of CTLA-4, IDO-1 and Foxp3 mRNA and an increase in the expression of granzyme B.

Total RNA was isolated from tumor tissue and analyzed for the expression of (A) CTLA-4, (B) granzyme B, (C) Foxp3, and (D) IDO-1 using specific probes. The results are shown as fold change in the level of specific mRNA relative to control and expressed as mean $\pm$ SEM for $n=3$. (E) Whole cell lysates were prepared from isolated melanomas and analyzed by Western blot for the expression IDO1 using a specific antibody with β -actin as loading control.

Figure 4: Effect of X4-136 with various immune checkpoint inhibitors on Akt/FOXO-3a pathway.

Whole cell lysates were prepared from isolated melanomas or treated cells and probed for the expression of various protein using specific antibodies. β -actin was used as loading control.

Figure 5: Effect of X4-136 and axitinib in the Renca-DM RCC model.

(A) Mice were treated with X4-136, axitinib alone and in combination as depicted. Tumor growth was plotted as mean $\pm$ SEM for $n=5-6$ for each treatment group. Tumors were harvested after 8 days of treatment. Single cell suspensions were analyzed by flow cytometry for (B) CD3⁺ cells, (C) CD8⁺ cells, (D) CD8⁺ perforin⁺ lymphocytes, (E) myeloid-derived suppressor cells (MDSC), and (F) regulatory T-cells (Tregs). The results are shown as fold change in number of cells relative to control and expressed as mean $\pm$ SEM for $n=5-6$.