International Immunopharmacology 75 (2019) 105818

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

SDF-1/CXCR4 axis facilitates myeloid-derived suppressor cells accumulation in osteosarcoma microenvironment and blunts the response to anti-PD-1 therapy

Kuo Jiang[a,1], Jia Li[b,1], Jitao Zhang[a,1], Lei Wang[c], Qianfeng Zhang[b], Junli Ge[b], Yunshan Guo[a], Biao Wang[a], Yi Huang[a], Tuanmin Yang[d], Dingjun Hao[a,*], Lequn Shan[a,*]

[a] Department of Spine Surgery, Honghui Hospital, Xi'an Jiaotong University, Xi'an, China
[b] Department of Obstetrics and Gynecology, Xijing Hospital, Fourth Military Medical University, Xi'an, China
[c] State Key Laboratory of Cancer Biology, Department of Biochemistry and Molecular Biology, Fourth Military Medical University, Xi'an, China
[d] Department of Bone Disease and Tumor, Honghui Hospital, Xi'an Jiaotong University, Xi'an, China

ARTICLE INFO

Keywords:
Immunotherapy
osteosarcoma
MDSCs
CXCR4

ABSTRACT

Immune checkpoint inhibitors, such as anti-PD-1/PD-L1, are a novel class of inhibitors that function as a tumor suppressing factor via modulation of immune cell-tumor cell interaction. To date, PD-1/PD-L1 inhibitors have been approved for the treatment of specific types of tumors and obtained good clinical efficacy. However, patients with osteosarcoma showed poor response to anti-PD-1/PD-L1 therapy, the mechanism of which is not well understood. In this study, we found that osteosarcoma tissues were heavily infiltrated by myeloid-derived suppressor cells (MDSCs) which could inhibit cytotoxicity T cell (CTL) expansion. Further study revealed that the vast majority of tumor-infiltrating MDSCs were CXCR4 positive and could migrate toward an SDF-1 gradient. The binding of SDF-1 to its receptor CXCR4 results in the activation of downstream AKT pathway that mediates reduced apoptosis of MDSCs. We also demonstrated that AMD3100, a CXCR4 antagonist, has a synergistic effect with anti-PD-1 antibody in tumor treatment in a murine model of osteosarcoma. These findings provide the basis for establishing CXCR4 antagonist and PD-1/PD-L1 inhibitors co-administration as a novel therapeutic regimen for patients with osteosarcoma and hold great promise for improving the therapeutic effect of osteosarcoma.

1. Introduction

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents, featured by rapid progression and poor prognosis. Osteosarcoma is highly metastatic and most patients have micro-metastases at the time of diagnosis [1]. Although the 5-year survival rate of osteosarcoma patients is increased to 65% by chemotherapy combined with surgery, the survival rate of osteosarcoma patients has not been significantly improved in the past 30 years. Moreover, current therapies have limited effects on the treatment of recurrent or metastatic osteosarcoma [2]. Osteosarcoma is still one of the most common malignant diseases that seriously endangers the health of adolescents. Therefore, it is particularly important to further explore the mechanisms underlying osteosarcoma development and to seek new approaches to treat osteosarcoma.

Immune checkpoint pathways, which normally maintain self-tolerance and reduce collateral tissue damage during anti-microbial immune responses, can be co-opted by cancer to evade immune destruction [3]. In the past decade, checkpoint inhibitor-based immunotherapy attracted much attention and has gradually become the focus of the field of cancer treatment. Among these, monoclonal antibodies (mAbs) are already being used to block the programmed death-1/programmed death ligand-1 (PD-1/PD-L1) pathway to treat human cancers, especially advanced solid tumors [4]. In the tumor microenvironment, the interaction of PD-L1 on the tumor cells with PD-1 on a T cell reduces T-cell function signals to prevent the immune system from attacking the tumor cells [5]. Use of an inhibitor that blocks the interaction of PD-L1 with the PD-1 receptor can promote the recovery of cytotoxicity T cells (CTLs) from inactivation, thus recognizing and killing tumor cells [5]. To date, a variety of PD-1/PD-L1 inhibitors have been approved for the treatment of specific types of tumors and achieved good curative effects, such as melanoma, non-small cell lung

* Corresponding authors.
E-mail addresses: haodingjun18@yeah.net (D. Hao), shanlequn@yeah.net (L. Shan).
[1] These authors have contributed equally to this work.

https://doi.org/10.1016/j.intimp.2019.105818
Received 22 June 2019; Received in revised form 7 August 2019; Accepted 9 August 2019
Available online 19 August 2019

cancer (NSCLC) and Hodgkin lymphoma [6–8]. However, anti-osteosarcoma drug testing using PD-1/PD-L1 inhibitors still lagged behind. Pre-clinical study showed that osteosarcoma is insensitive to anti-PD-1 monotherapy [9]. So, it is of great value to study the mechanism of drug resistance to therapeutic PD-1 blockade in osteosarcoma and to increase the sensitivity of osteosarcoma tissue to anti-PD-1 mAbs.

The presence of large amounts of activated CTLs in tumor tissue is an important factor in evaluating the efficacy of anti-PD-1 therapy [10]. Recent studies found that myeloid-derived suppressor cells (MDSCs), which are derived from bone marrow progenitor cells and immature myeloid cells, can also promote the development of tumors by inhibiting anti-tumor immune responses and remodeling the tumor microenvironment [11]. MDSCs not only inhibit the trafficking of CTLs into the tumors, but also remove the key nutrients necessary for T cell proliferation by releasing reactive oxygen species (ROS), thus suppressing T cell function [11]. In the presence of MDSCs, even if the disabled CTLs were activated by PD-1/PD-L1 antibody, the tumor immune evasion still occurs due to the insufficient infiltration of CTLs in the tumors [12].

In this study, we sought to delve deeper into the MDSCs-mediated mechanism by which osteosarcoma patients show poor response to anti-PD-1 therapy. We found that lots of CTL-inhibiting MDSCs migrate into the osteosarcoma microenvironment through a SDF-1 (CXCL12)/CXCR4 axis. The binding of SDF-1 to its receptor CXCR4 results in the activation of downstream AKT pathway that mediates reduced apoptosis of MDSCs. We also demonstrated that AMD3100, a CXCR4 antagonist [13], has a synergistic effect with anti-PD-1 antibody in tumor treatment in a murine model of osteosarcoma. These findings provide the basis for establishing CXCR4 antagonist and PD-1/PD-L1 inhibitors co-administration as a novel therapeutic regimen for patients with osteosarcoma and hold great promise for improving the therapeutic effect of osteosarcoma.

2. Results

2.1. CXCR4 is predominantly expressed in stromal cells and inversely correlated with CD8 T-cell infiltration in osteosarcoma

To investigate the mechanism of drug resistance to therapeutic PD-1 blockade in osteosarcoma, we first examined the expression of PD-L1 and CD8 in a cohort of 40 osteosarcoma tissues. The results showed that PD-L1 was widely expressed in osteosarcoma samples (35/40), with most of them exhibiting a homogeneous immunoreactivity inside the tumor (Fig. 1A). However, CD8 staining was rarely detected in osteosarcoma tissues (7/40), with only two samples showing a strong signal (Fig. 1A, B and data not shown). These findings support the notion that assessment of PD-L1 expression alone is inefficient in predicting response to anti-PD-1 immunotherapy in osteosarcoma, and other biomarkers such as CTL infiltration could be a key limiting factor for efficacious cancer immunotherapy [10,14]. Next, we examined the expression pattern of CD11b and CXCR4 in the same cohort, which have been reported to be involved in the process of CTL recruitment [11,15]. Results showed that both molecules were widely expressed in osteosarcoma samples (75% and 57.5%, respectively). Moreover, most CXCR4 positive tissues displayed $CD11b^+$ signal and CD8 T cells were largely absent in these CD11b positive tissues (Fig. 1A, B, C). Interestingly, we found that CXCR4 was predominantly expressed in the tumor stroma rather than the parenchyma (Fig. 1A), suggesting that it might play a major role in human osteosarcoma microenvironment. Kaplan-Meier's analysis revealed that those osteosarcoma patients bearing $CXCR4^+/CD8^-$ tumors had significantly shorter overall survival ($P < 0.05$) than patients bearing $CXCR4^-/CD8^+$ tumors (Fig. 1D). Taken together, these data indicated that CXCR4 blockade might have synergistic effects with anti-PD-1 antibody in the treatment of osteosarcoma.

2.2. PD-1 and CXCR4 combination blockade expands infiltrating CTLs and controls tumor growth in a murine model of osteosarcoma

We established a subcutaneous osteosarcoma model in immunocompetent BALB/c mice using luciferase-expressing syngeneic K7M2-luc cells [16]. Then, we tested the effect of AMD3100 and αPD-1 used alone or in combination on tumor growth and animal survival in osteosarcoma-bearing mice. In animals treated with αPD-1 (200 mg per mouse) alone, tumor growth was not suppressed compared with that in saline-treated mice, as recorded by bioluminescence signals (Fig. 2A, B). Nevertheless, treatment with AMD3100 (3 mg/kg) alone slightly inhibited tumor growth and prolonged mouse survival (Fig. 2A, B, C). Moreover, the combination treatment showed significantly improved antitumor efficacy on inhibition of tumor growth ($P < 0.05$, $P < 0.001$) and prolongation of mouse survival ($P < 0.01$, $P < 0.001$) compared with AMD3100 and αPD-1 monotherapy, respectively (Fig. 2A, B, C). Immunohistochemical staining and flow cytometric analysis of CD8 T lymphocytes showed that both AMD3100 monotherapy and combination treatment greatly facilitated effector T cell infiltration into the K7M2 osteosarcoma tumors (Fig. 2D, E). The earlier data indicate that PD-1/PD-L1 blockade could activate functionally disabled CD8 T cells. We, thus, tested whether the earlier changes were associated with activation of $CD8^+$ tumor-infiltrating lymphocytes (TILs) in addition to cell number expansion. We found that AMD3100 and αPD-1 co-administration caused a further upregulation of Ki67, intracellular granzyme B, IFN-γ, and TNF-α expression in $CD8^+$ TILs, compared with AMD3100 and αPD-1 monotherapy respectively (Fig. 2F), suggesting that CXCR4 antagonist had a synergistic effect with anti-PD-1 antibody in activating the CTLs within osteosarcoma.

2.3. Tumor-infiltrating MDSCs express high levels of CXCR4 and are diminished by AMD3100 administration in osteosarcoma

To explore the cellular mechanisms underlying CD8 T-cell expansion by CXCR4 antagonist, we examined the different immune cell subpopulations in the K7M2 tumors. We found that $CD11b^+/Gr\text{-}1^+$ MDSCs were the most abundant TILs within the saline-treated tumors and were significantly diminished by both AMD3100 monotherapy and combination treatment (Fig. 3A, B). However, treatment with αPD-1 alone had no effect on the abundance of tumor-infiltrating MDSCs (Fig. 3A, B), which was consistent with our previous findings showing that CD8 T-cell infiltration was not affected by αPD-1 monotherapy (Fig. 2D, E). Furthermore, we examined the expression levels of CXCR4 on MDSCs using flow cytometric analysis. Results showed that the vast majority of tumor-infiltrating MDSCs were CXCR4 positive and expressed higher levels of CXCR4 than those of splenic MDSCs (Fig. 3C, D). Then, we sorted the $CD11b^+/Gr\text{-}1^+$ subsets from the K7M2 tumors and evaluated their capacity to suppress proliferation of anti-CD3/anti-CD28-activated splenic T cells. At MDSC/T cell ratios of 1:2, intra-tumoral MDSCs inhibited T cell proliferation by about 80% and remained highly suppressive even at MDSC/T cell ratios of 1:8. Although splenic MDSCs also suppressed T cell proliferation, they were less potent than intra-tumoral MDSCs and their suppressive potential was more readily diminished with decreasing MDSC/T cell ratios (Fig. 3F and data not shown). Overall, these data indicated that AMD3100 might facilitate CD8 T-cell expansion by diminishing CXCR4-expressing MDSCs within the K7M2 osteosarcoma tumors.

2.4. CXCL12/CXCR4 axis promotes migration and survival of MDSCs in osteosarcoma

We next sought to investigate the mechanisms underlying MDSC reduction by CXCR4 antagonist. To this end, we sorted the $CD11b^+/Gr\text{-}1^+$ MDSCs from the K7M2 tumors and performed transwell chemotaxis assays. The results showed that, CXCL12, which is the only known ligand for CXCR4 [17], greatly enhanced cell migration ability of MDSCs

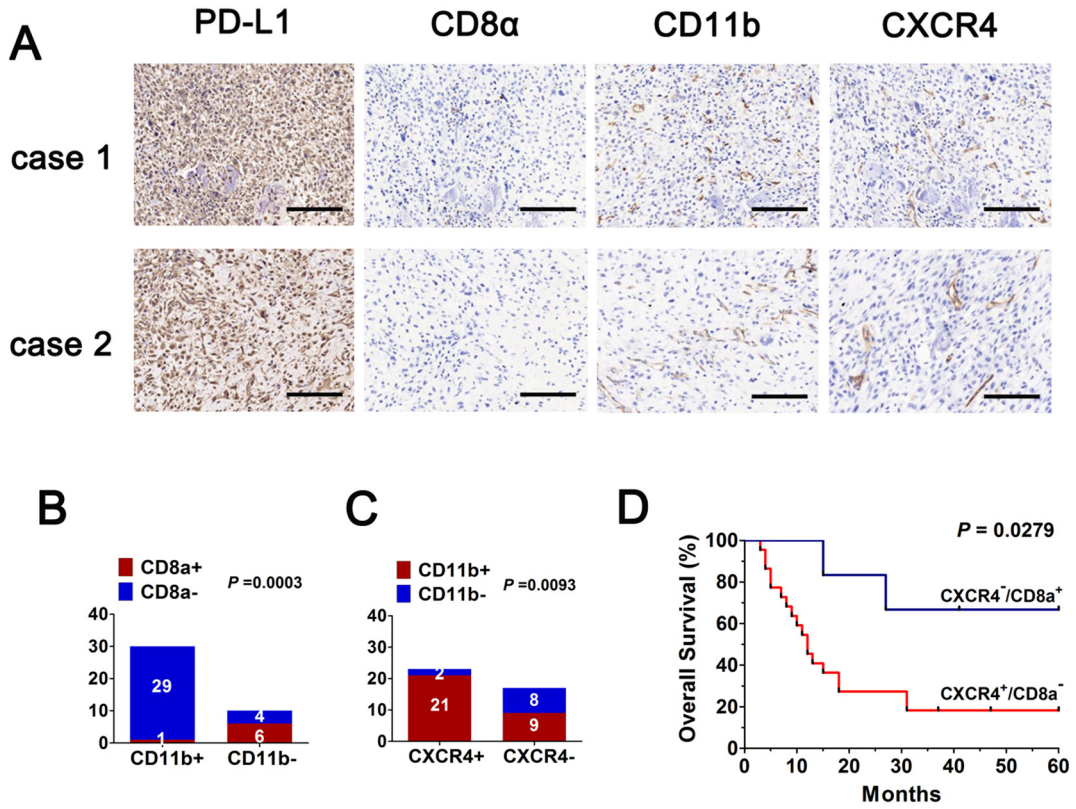

Fig. 1. CXCR4 is predominantly expressed in stromal cells and inversely correlated with CTL infiltration in osteosarcoma. (A) Representative IHC staining of PD-L1, CD8α, CD11b and CXCR4 in consecutive tissue microarray slides consisted of 40 osteosarcoma samples. Scale bar: 100 μm. Statistical comparisons were performed using Fisher's exact test (B and C). (D) Kaplan-Meier curves representing the probabilities of overall survival in 40 osteosarcoma patients stratified according to the expression levels of CXCR4 and CD8α.

(Fig. 4A). However, this effect was completely abolished by AMD3100 pretreatment, suggesting that AMD3100 could impair the CXCL12-induced chemotaxis of MDSCs in osteosarcoma (Fig. 4A). Both immunoblot detection of active Caspase-3 and Annexin V/propidium iodide assay detected by flow cytometry showed that CXCL12 administration also remarkably inhibited apoptosis of MDSCs, while pretreatment with AMD3100 largely abrogated this inhibitory effect (Fig. 4B, C). Previous studies demonstrated that CXCL12 regulates chemotaxis and survival through PI3K/Akt signaling pathway in various cell types [17–19]. In line with this, our experiments also found that CXCL12 treatment caused significant activation of Akt in MDSCs, which could be blocked by AMD3100 (Fig. 4D). Taken together, these data suggested that the CXCL12/CXCR4 axis might promote migration and survival of MDSCs via activating PI3K/Akt pathway in osteosarcoma.

3. Discussion

At present, a variety of PD-1/PD-L1 inhibitors have been approved for the treatment of specific types of tumors, including melanoma, NSCLC, advanced Hodgkin lymphoma and so on [6–8]. In a phase III randomized clinical trial of nivolumab (anti-PD-1 monoclonal antibody) in patients with melanoma, Weber et al. found that the median relapse-free survival (RFS) time of advanced melanoma patients treated with nivolumab alone was more than twice that of ipilimumab alone (6.9 months vs 2.9 months) [20]. They also found that melanoma patients benefited even more when nivolumab was used in combination with ipilimumab (11.5 months). In the clinical study of NSCLC, anti-PD-1 antibody also showed great potential. The total remission rate of NSCLC patients treated with nivolumab was 17%, and the remission duration was > 18 months [21]. However, anti-osteosarcoma drug testing using PD-1/PD-L1 inhibitors still lagged behind. Pre-clinical study showed that osteosarcoma was insensitive to anti-PD-1 monotherapy [9]. An animal experiment using anti-PD-L1 mAb in the treatment of metastatic osteosarcoma showed that prolonging the duration of treatment with anti-PD-L1 mAb did not make mice survive longer, and additional dose of anti-PD-L1 mAb also failed to achieve survival benefits [22]. In this study, we found that osteosarcoma tissues are poorly infiltrated by CD8 T lymphocytes (Fig. 1). In this case, even if the disabled CTLs were activated by anti-PD-1 antibody, CTLs are still inaccessible to cancer cells. Because CTLs kills cancer cells by directly contacting them and releasing perforin and granzyme B [23], it is critical to develop optimal combination immunotherapies which can increase CTL infiltration in osteosarcoma tumors.

Myeloid-derived suppressor cells, which are derived from bone marrow progenitor cells and immature myeloid cells, can also promote the development of tumors by inhibiting anti-tumor immune responses and remodeling the tumor microenvironment [11]. When MDSCs are recruited into tumor tissues, they upregulate PD-L1 expression on their cell membranes and recruit regulatory T cells (Treg) into the tumors by secreting chemokines such as CCL4 and CCL5, to enhance the effect of immunosuppression. MDSCs not only inhibit the trafficking of CTLs into the tumors, but also remove the key nutrients necessary for T cell proliferation by releasing ROS, thus suppressing T cell function [11]. In a murine model of metastatic osteosarcoma, Horlad et al. found that there were large numbers of MDSCs in osteosarcoma tissues and these MDSCs play an important role in driving osteosarcoma development and metastasis [24]. In our work, we found that osteosarcoma tissues were heavily infiltrated by MDSCs which could inhibit T cell expansion. This finding suggested that clearance of MDSCs might facilitate the efficacy of anti-PD-1 therapy in the treatment of osteosarcoma.

CXCR4, which was reported to be widely expressed in tumor

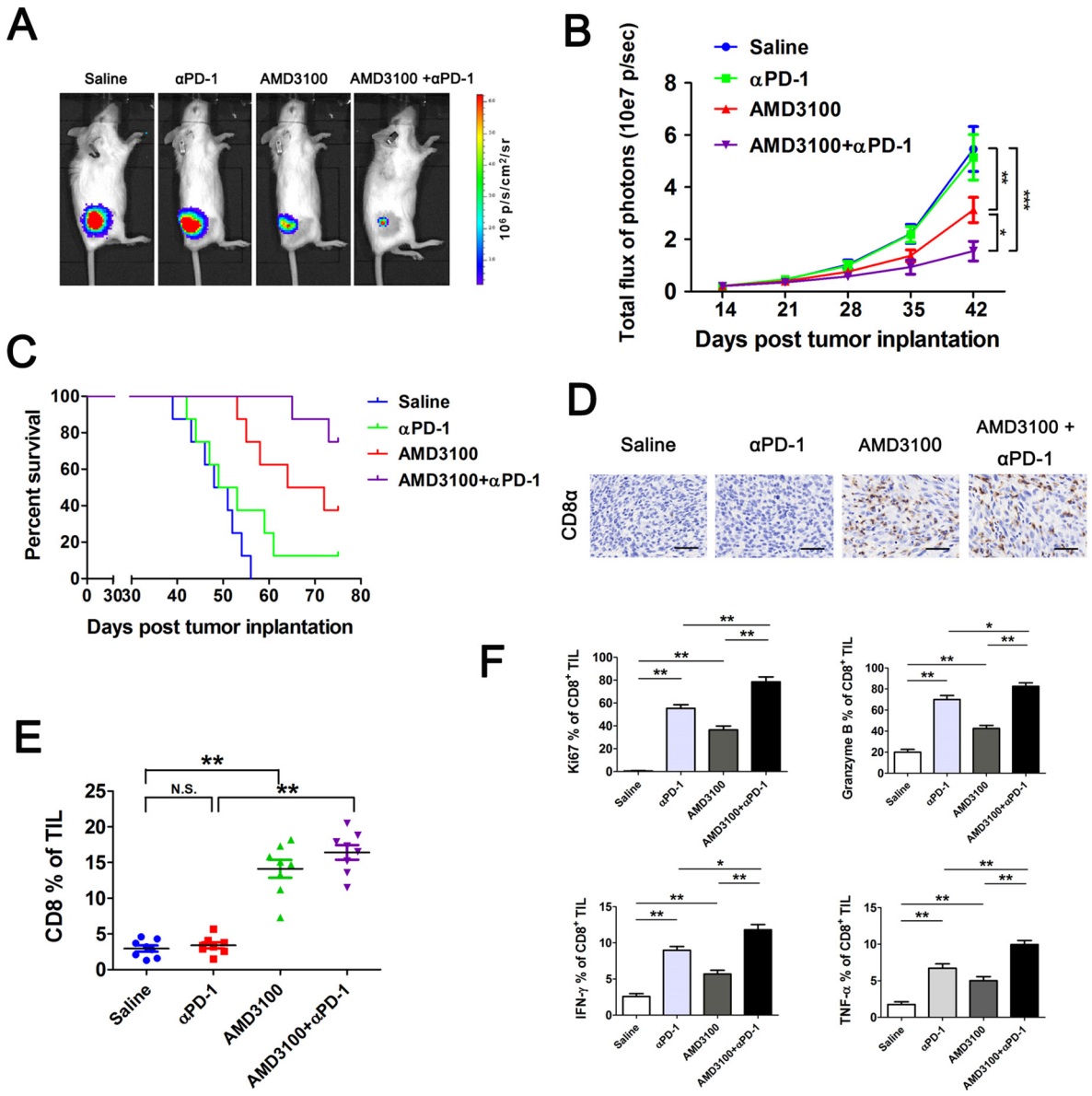

Fig. 2. PD-1 and CXCR4 combination blockade expands infiltrating CTLs and controls tumor growth in an osteosarcoma mouse model. (A and B) Bioluminescence imaging in a subcutaneous transplanted model of K7M2 mouse osteosarcoma cells. 20 days after cell injection, tumor-bearing mice were treated with AMD3100 (3 mg/kg, every other day I.P.), anti-PD1 antibody (200 μg per mouse, every three days I.P.) or the combination, for a total of 20 days (n = 8 per group). Representative images were taken at the end of the treatments (A) and tumor growth was assessed weekly by bioluminescent imaging, as measured by photon emission (B). (C) Kaplan-Meier curves showing the survival time of BALB/c mice in figure B. (D) Representative IHC staining of CD8α in tumor tissues isolated from the mice in figure A. (E) Percentages of $CD8^+$ CTLs in intratumoral $CD45^+$ cell population, measured by flow cytometry. (F) Percentages of $Ki67^+$, IFN-γ^+, TNF-α^+, and Granzyme B^+ cells in intratumoral $CD8^+$ cell population, measured by flow cytometry.

parenchymal cells [25], was found to be predominantly expressed in the stromal cells within osteosarcoma in our study (Fig. 1). Further investigation showed that vast majority of tumor-infiltrating MDSCs were CXCR4 positive and expressed higher levels of CXCR4 than those of splenic MDSCs (Fig. 3). Therefore, we supposed that CXCR4 antagonist might have a synergistic effect with anti-PD-1 antibody by diminishing the MDSCs within osteosarcoma tissues. Previous studies using AMD3100 as a CXCR4 antagonist have demonstrated that this small molecule inhibitor holds great promise in the treatment of osteosarcoma. Liao et al. found that AMD3100 reduces CXCR4-mediated survival and metastasis of osteosarcoma by inhibiting JNK and Akt, but not p38 or Erk1/2, pathways in in vitro and mouse experiments [26]. In another study, Jiang et al. found that AMD3100 combined with triptolide, can reduce proliferation and metastasis, and induce apoptosis of U2OS osteosarcoma cells, which may be related to the Erk1/2, Akt, STAT3 and NF-κB pathways [27]. In our work, we found for the first time that AMD3100 could also remodel the osteosarcoma microenvironment by targeting immunosuppressive cells. This is largely due to the fact that CXCR4 was predominantly expressed in the stromal cells within osteosarcoma, such as CTL-inhibiting MDSCs. AMD3100 administration significantly reduced the CXCR4-expressing MDSCs by impairing their chemotaxis to CXCL12 and promoting apoptosis, facilitating effector T cell infiltration into the K7M2 osteosarcoma tumors. In conclusion, our findings provide the basis for establishing CXCR4 antagonist and PD-1/PD-L1 inhibitors co-administration as a novel therapeutic regimen for patients with osteosarcoma and hold great promise for improving the therapeutic effect of osteosarcoma.

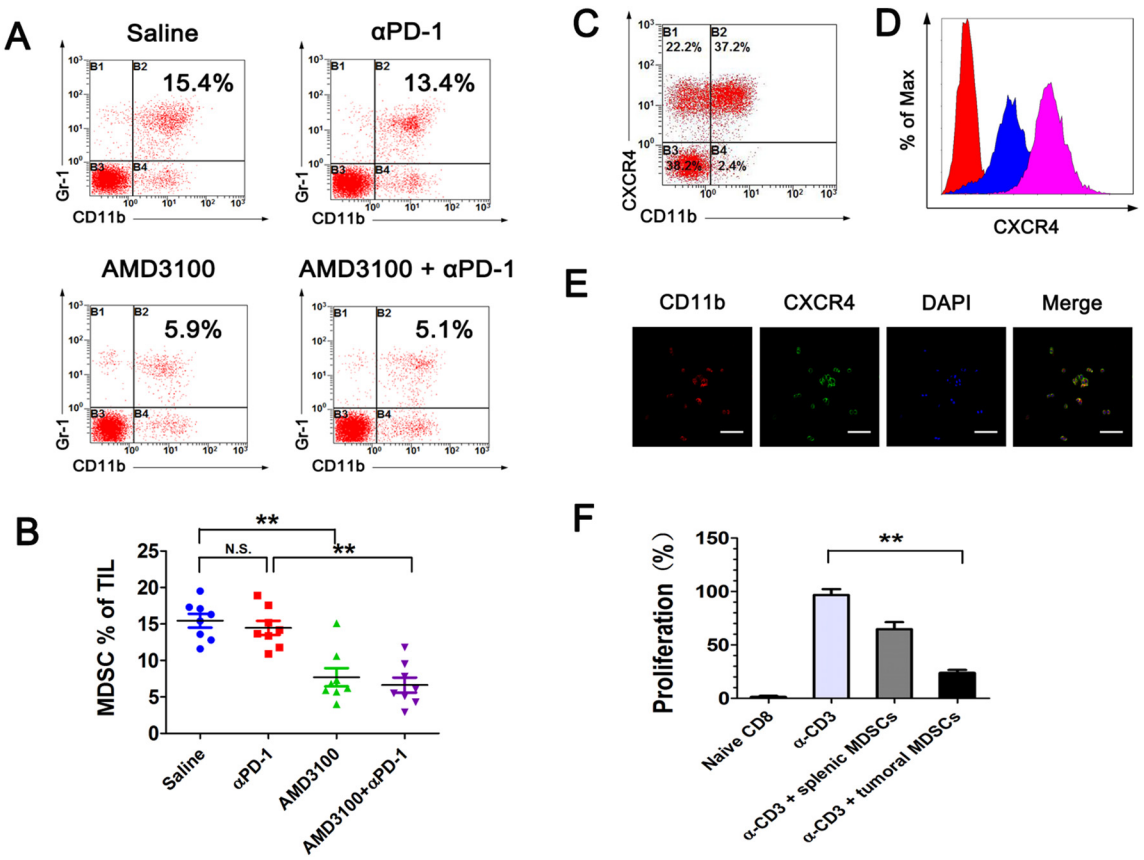

Fig. 3. Tumor-infiltrating MDSCs express high levels of CXCR4 and are diminished by AMD3100 administration in osteosarcoma. (A and B) Percentages of Gr-1$^+$/CD11b$^+$ MDSCs in intratumoral CD45$^+$ cell population in different treatment groups, measured by flow cytometry (A). (C) Percentages of CXCR4$^+$ MDSCs in intratumoral Gr-1$^+$/CD11b$^+$ cell population. (D) Expression levels of CXCR4 in splenic MDSCs (blue) and intratumoral MDSCs (purple), measured by flow cytometry. (E) Immunofluorescence microscopy analysis of CD11b and CXCR4 in FACS-sorted MDSCs. (F) Splenic MDSCs and intratumoral MDSCs were placed in a proliferation assay with CFSE-labeled CD8$^+$ T cells at the designated MDSC/T cell ratios. Anti-CD3/CD28 beads were used at a 1:2 ratio with T cells to induce proliferation. Cells were harvested on day 5 and analyzed by FACS for Violet dilution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Materials and methods

4.1. Mice and tumor models

Six-week old female BALB/cJ mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and maintained under specific pathogen-free conditions. Mice had free access to food and water during the whole experimental period. The K7M2 mouse osteosarcoma cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences and 5×10^6 cells were injected into BALB/cJ mice subcutaneously. AMD3100 was administered intraperitoneally 10 times every other day from d20 post-tumor implantation dosing at 3 mg/kg in 100 μl. αPD-1 was applied at 200 μg/100 μl per mouse every three days starting from d22 for 6 doses. Tumor growth were assessed weekly by bioluminescent imaging on the Xenogen In Vivo Imaging System (IVIS, Caliper Life Science, Hopkinton, MA). All animal experiments were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

4.2. Reagents and antibodies

CXCR4 inhibitor, Plerixafor (AMD3100), was purchased form Selleck Chemicals (Houston, TX, USA). Recombinant murine SDF-1 (CXCL12) was obtained from R&D Systems (Minneapolis, MN). InVivoMab anti-mouse PD-1 was purchased from Bio X Cell (West Lebanon, NH, USA); anti-PD-L1, anti-CD8α, anti-Akt, anti-pAkt antibodies were purchased from Cell Signaling Technology (Beverly, MA); anti-CXCR4, anti-CD11b antibodies were purchased from Abcam (Cambridge, MA); anti-Gr-1, anti-IFN-γ, anti-TNF-α antibodies were purchased from eBioscience (San Diego, USA).

4.3. MDSC isolation from tumors and spleens

Solid tumors were dissected and mechanically dissociated into small fragments (< 4 mm) with a scalpel, followed by digestion with mouse tumor dissociation kit (Miltenyi Biotec) for 40 min at 37 °C. After single-cell suspensions were obtained, erythrocytes were removed by red blood cell lysis buffer (ACK) and dead cells were depleted with a dead cell removal kit (Miltenyi Biotec). Splenic single-cell suspensions were prepared by mechanical dissociation, followed by removal of red blood cells with ACK lysis buffer. MDSCs were further isolated by cell sorting on BD FACSAria II cell sorter after incubating with FITC-conjugated anti-Gr-1 and PE-conjugated anti-CD11b antibodies.

4.4. T-cell suppression and MDSC migration assay

CD8$^+$ T cells were isolated by using either biotinylated or PE-conjugated anti-CD8α antibody and corresponding streptavidin or anti-PE microbeads on MACS LS columns according to the manufacturer's protocol (Miltenyi Biotec). T-cell suppression assay was performed using FACS-sorted MDSCs and CFSE (Invitrogen)-labeled CD8$^+$ T cells in anti-CD3- and anti-CD28-coated 96-well plates at an MDSC/T-cell ratio of 0:1, 1:1, 1:2, 1:4, 1:8, with 1.0×10^4 to 8.0×10^4 MDSCs used in each ratio. Cells were analyzed after 72 h by flow cytometry, and the

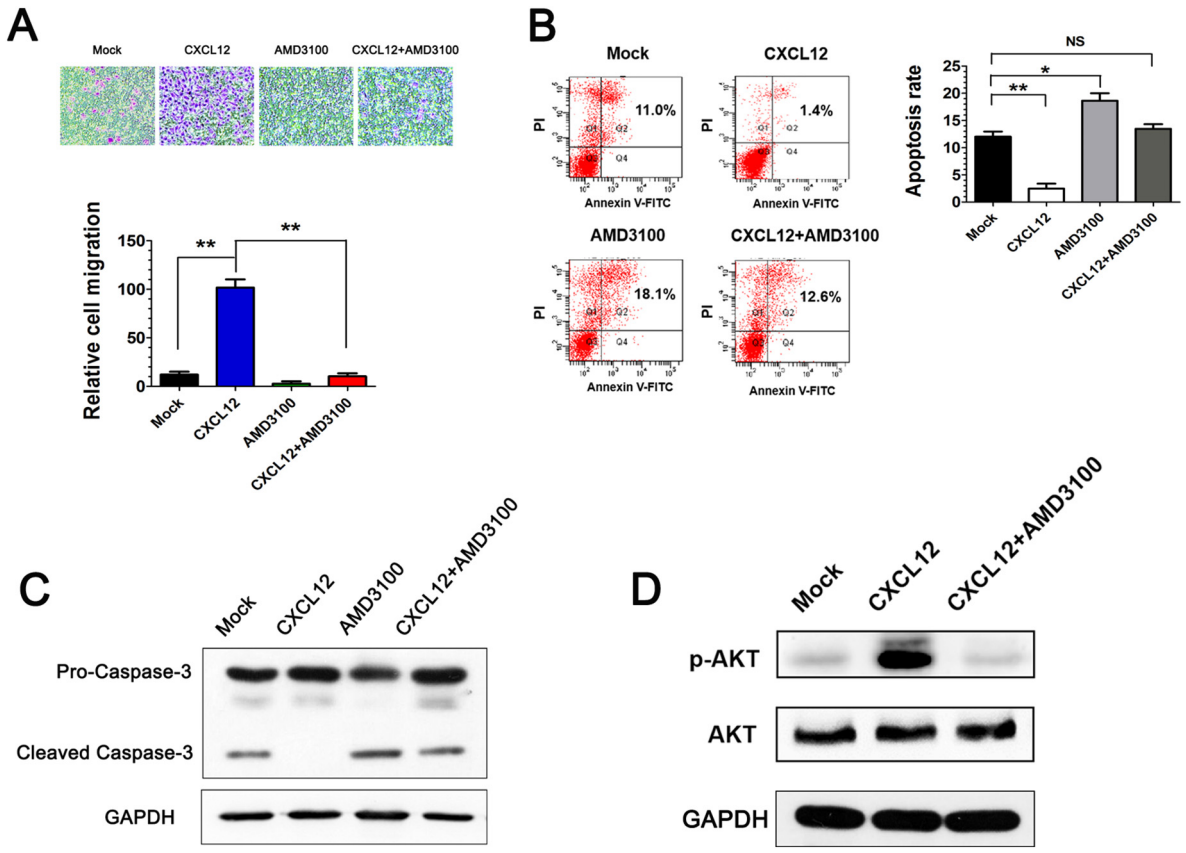

Fig. 4. CXCL12/CXCR4 axis promotes migration and survival of MDSCs in osteosarcoma. (A) Transwell migration assay of MDSCs untreated or pretreated with AMD3100, CXCL12 was used as a chemoattractant. (B) Left panel: Representative flow cytometry plots of Annexin V/PI-stained MDSCs, untreated or treated with CXCL12, AMD3100, or both; right panel: Histograms depicting the apoptotic rates of MDSCs. (C) Western blot analysis of the levels of cleaved Caspase-3 in MDSCs, untreated or treated with CXCL12, AMD3100, or both. (D) Western blot analysis of the levels of phosphorylated Akt in MDSCs, untreated or treated with CXCL12, AMD3100, or both.

suppression of T cells is calculated as described previously [28]. For the MDSC migration assay, an equal number of FACS-sorted MDSCs, untreated or pretreated with AMD3100, were placed on the top chamber of a transwell system (BD Falcon), and medium supplemented with SDF-1 was used as a chemoattractant in the lower chamber. Cells were allowed to migrate to the bottom well for 6 h at 37 °C with 5% CO_2 and cells on the lower surface of the membrane were stained with crystal violet, air dried and photographed.

4.5. Flow cytometry analysis

Flow cytometry was performed using a FACS LSR-II (BD). Data were further analyzed by FACS DIVA 7.0 (BD) or FlowJo 7.6.5 software (Tree Star).

4.6. Immunohistochemistry (IHC) and immunoblotting

IHC staining in tissue samples and Western blotting was performed as described previously [29].

4.7. Statistical analysis

Statistical analysis was performed using SPSS 11.0 for Windows. All data were presented as mean ± SEM. Overall survival was analyzed with the Kaplan-Meier method and the statistical probability (P-value) was generated by log-rank test. Two-tailed Student's *t*-test was used to evaluate the statistical significance of differences between two groups of data in T-cell suppression assay, Annexin-V assay and transwell migration assay. Differences were considered significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

Declaration of Competing Interest

No potential conflicts of interest were disclosed.

Acknowledgements

This study was supported by the National Natural Science Foundation of China [81702668, 81402133, 81602517, 81802167 and 30901784], and the Fundamental Research Funds of Central Universities (xzy012019123).

References

[1] M.S. Isakoff, S.S. Bielack, P. Meltzer, R. Gorlick, Osteosarcoma: current treatment and a collaborative pathway to success, J. Clin. Oncol. 33 (27) (2015) 3029–3035.
[2] D.J. Harrison, D.S. Geller, J.D. Gill, V.O. Lewis, R. Gorlick, Current and future therapeutic approaches for osteosarcoma, Expert. Rev. Anticancer. Ther. 18 (1) (2018) 39–50.
[3] S.L. Topalian, C.G. Drake, D.M. Pardoll, Immune checkpoint blockade: a common denominator approach to cancer therapy, Cancer Cell 27 (4) (2015) 450–461.
[4] L. Chen, X. Han, Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future, J. Clin. Invest. 125 (9) (2015) 3384–3391.
[5] N.L. Syn, M. Teng, T. Mok, R.A. Soo, De-novo and acquired resistance to immune checkpoint targeting, Lancet Oncol. 18 (12) (2017) e731–e741.
[6] S.M. Ansell, A.M. Lesokhin, I. Borrello, A. Halwani, E.C. Scott, M. Gutierrez, et al., PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma, N. Engl. J. Med. 372 (4) (2015) 311–319.
[7] E.B. Garon, N.A. Rizvi, R. Hui, N. Leighl, A.S. Balmanoukian, J.P. Eder, et al., Pembrolizumab for the treatment of non-small-cell lung cancer, N. Engl. J. Med. 372 (21) (2015) 2018–2028.
[8] C. Robert, J. Schachter, G.V. Long, A. Arance, J.J. Grob, L. Mortier, et al.,

Pembrolizumab versus ipilimumab in advanced melanoma, N. Engl. J. Med. 372 (26) (2015) 2521–2532.
[9] P. Thanindratarn, D.C. Dean, S.D. Nelson, F.J. Hornicek, Z. Duan, Advances in immune checkpoint inhibitors for bone sarcoma therapy, J. Bone Oncol. 15 (2019) 100221.
[10] I. Melero, A. Rouzaut, G.T. Motz, G. Coukos, T-cell and NK-cell infiltration into solid tumors: a key limiting factor for efficacious cancer immunotherapy, Cancer Discov. 4 (5) (2014) 522–526.
[11] V. Kumar, S. Patel, E. Tcyganov, D.I. Gabrilovich, The nature of myeloid-derived suppressor cells in the tumor microenvironment, Trends Immunol. 37 (3) (2016) 208–220.
[12] J. Duraiswamy, G.J. Freeman, G. Coukos, Therapeutic PD-1 pathway blockade augments with other modalities of immunotherapy T-cell function to prevent immune decline in ovarian cancer, Cancer Res. 73 (23) (2013) 6900–6912.
[13] G.A. Donzella, D. Schols, S.W. Lin, J.A. Este, K.A. Nagashima, P.J. Maddon, et al., AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor, Nat. Med. 4 (1) (1998) 72–77.
[14] M.R. Junttila, F.J. de Sauvage, Influence of tumour micro-environment heterogeneity on therapeutic response, NATURE 501 (7467) (2013) 346–354.
[15] T. Zhang, R. Somasundaram, K. Berencsi, L. Caputo, P. Rani, D. Guerry, et al., CXC chemokine ligand 12 (stromal cell-derived factor 1 alpha) and CXCR4-dependent migration of CTLs toward melanoma cells in organotypic culture, J. Immunol. 174 (9) (2005) 5856–5863.
[16] E.T. Ek, C.R. Dass, P.F. Choong, Commonly used mouse models of osteosarcoma, Crit. Rev. Oncol. Hematol. 60 (1) (2006) 1–8.
[17] B.A. Teicher, S.P. Fricker, CXCL12 (SDF-1)/CXCR4 pathway in cancer, Clin. Cancer Res. 16 (11) (2010) 2927–2931.
[18] T. Yano, Z. Liu, J. Donovan, M.K. Thomas, J.F. Habener, Stromal cell derived factor-1 (SDF-1)/CXCL12 attenuates diabetes in mice and promotes pancreatic beta-cell survival by activation of the prosurvival kinase Akt, DIABETES 56 (12) (2007) 2946–2957.
[19] M. Zhao, B.M. Mueller, R.G. DiScipio, I.U. Schraufstatter, Akt plays an important role in breast cancer cell chemotaxis to CXCL12, Breast Cancer Res. Treat. 110 (2) (2008) 211–222.
[20] J.S. Weber, R.R. Kudchadkar, B. Yu, D. Gallenstein, C.E. Horak, H.D. Inzunza, et al., Safety, efficacy, and biomarkers of nivolumab with vaccine in ipilimumab-refractory or -naive melanoma, J. Clin. Oncol. 31 (34) (2013) 4311–4318.
[21] R. Sundar, B.C. Cho, J.R. Brahmer, R.A. Soo, Nivolumab in NSCLC: latest evidence and clinical potential, Ther. Adv. Med. Oncol. 7 (2) (2015) 85–96.
[22] D.M. Lussier, J.L. Johnson, P. Hingorani, J.N. Blattman, Combination immunotherapy with alpha-CTLA-4 and alpha-PD-L1 antibody blockade prevents immune escape and leads to complete control of metastatic osteosarcoma, J. Immunother. Cancer 3 (2015) 21.
[23] J.A. Trapani, M.J. Smyth, Functional significance of the perforin/granzyme cell death pathway, Nat. Rev. Immunol. 2 (10) (2002) 735–747.
[24] H. Horlad, Y. Fujiwara, K. Takemura, K. Ohnishi, T. Ikeda, H. Tsukamoto, et al., Corosolic acid impairs tumor development and lung metastasis by inhibiting the immunosuppressive activity of myeloid-derived suppressor cells, Mol. Nutr. Food Res. 57 (6) (2013) 1046–1054.
[25] F. Balkwill, The significance of cancer cell expression of the chemokine receptor CXCR4, Semin. Cancer Biol. 14 (3) (2004) 171–179.
[26] Y.X. Liao, Z.Z. Fu, C.H. Zhou, L.C. Shan, Z.Y. Wang, F. Yin, et al., AMD3100 reduces CXCR4-mediated survival and metastasis of osteosarcoma by inhibiting JNK and Akt, but not p38 or Erk1/2, pathways in in vitro and mouse experiments, Oncol. Rep. 34 (1) (2015) 33–42.
[27] C. Jiang, X. Fang, H. Zhang, X. Wang, M. Li, W. Jiang, et al., AMD3100 combined with triptolide inhibit proliferation, invasion and metastasis and induce apoptosis of human U2OS osteosarcoma cells, Biomed. Pharmacother. 86 (2017) 677–685.
[28] M. Battaglia, A. Stabilini, M.G. Roncarolo, Rapamycin selectively expands CD4 + CD25 + FoxP3 + regulatory T cells, BLOOD 105 (12) (2005) 4743–4748.
[29] L. Wang, X. Zhang, L.T. Jia, S.J. Hu, J. Zhao, J.D. Yang, et al., c-Myc-mediated epigenetic silencing of MicroRNA-101 contributes to dysregulation of multiple pathways in hepatocellular carcinoma, HEPATOLOGY 59 (5) (2014) 1850–1863.