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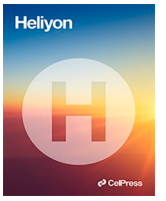

Research article

CXCL6-CXCR2 axis-mediated PD-L2^{+} mast cell accumulation shapes the immunosuppressive microenvironment in osteosarcoma

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ABSTRACT

Osteosarcoma (OS) is the most common primary bone malignancy and has a high propensity for local invasion and metastasis. The tumour microenvironment of OS is infiltrated by a large number of immune cells, which play a crucial role in its progression and prognosis. Mast cells are important innate immune cells in the tumour stroma and exhibit different phenotypes in diverse tumour microenvironments. However, the underlying mechanisms of mast cell accumulation and the phenotypic characteristics of mast cells in OS remain poorly understood. In this article, we found for the first time that mast cell accumulation in osteosarcoma tissue was modulated by the CXCL6-CXCR2 axis and that the number of infiltrating mast cells was significantly greater in tumour tissues than in adjacent nontumour tissues. These tumour-infiltrating mast cells express high levels of the immunosuppressive molecule PD-L2, and survival analyses revealed that patients in the PD-L2^{+} high-expression group had a worse prognosis. *In vitro*, mast cells were induced to express PD-L2 in a time- and dose-dependent manner using OS tissue culture supernatants to mimic the tumour microenvironment. Mechanistic studies revealed that tumour cell-derived G-CSF significantly induced mast cell PD-L2 expression by activating STAT3. Importantly, mast cells overexpressing PD-L2 inhibit tumour-specific CD8^{+} T-cell proliferation and tumour-killing cytokine secretion, which is reversed by blocking PD-L2 on mast cells. Therefore, our findings provide new insight into the immunosuppressive and tumorigenic roles of mast cells, as well as a novel mechanism by which PD-L2-expressing mast cells mediate immune tolerance.

1. Introduction

Osteosarcoma (OS), the most common primary malignant neoplasm of the skeletal system, predominantly affects the paediatric and adolescent population and is often fatal due to its high propensity to metastasize to the pulmonary region [1]. Approximately 4.8 patients per million worldwide are diagnosed with OS, which poses a substantial economic and health burden [2]. Despite the current available treatments of surgery and intensive chemotherapy, the 5-year survival rate for recurrent or metastatic OS is less than 25 % [3]. To improve the prognosis of patients with OS, future treatments that are effective and safe and exhibit minimal cytotoxicity to

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healthy tissues must be developed.

It has been accepted that cross-talk between the tumour and the immune system in the tumour microenvironment influences the development and prognosis of OS [4,5], with the clinical outcomes of OS patients supporting the key role of adaptive immunity in predicting OS prognosis [6]. However, relatively little is known about the role of innate immunity and innate immune cells in OS progression. As innate immune cells, mast cells can exert profound immunoregulatory effects on tumour progression by modulating angiogenesis [7], interacting with other immune cells [8], and remodeling the tumour microenvironment [9]. Currently, a limited number of studies have been conducted on mast cells in OS, and most researchers have investigated the relationship between OS survival rates and mast cell infiltration using immunohistochemistry [10]. The phenotypes, regulatory mechanisms, and clinical correlations of mast cells in OS, as well as the precise relationships with other immune cells, remain unclear.

Chemokine receptors and ligands play important functions in recruiting immune immunocytes and remodeling the tumor microenvironment [11]. There is evidence that cytokines of the C-X-C motif chemokine ligand 6 (CXCL6) levels are elevated in the serum of OS patients, and that recombinant CXCL6 may promote the proliferation of OS cells [12]. As a result of its interaction with C-X-C motif chemokine receptor 1 (CXCR1) and CXCR2, CXCL6 regulates its downstream pathways [13]. It is speculated that CXCL6/CXCR2 axis plays an important role in the progression of OS.

In our study, the interaction between mast cells and T cells in OS microenvironments was investigated. We revealed that mast cell recruitment into the tumour microenvironment is modulated by CXCL6-CXCR2 signalling. Furthermore, granulocyte colony-stimulating factor (G-CSF) released from OS cells successfully induces the expression of programmed death-ligand 2 (PD-L2) on mast cells by activating signal transducer and activator of transcription 3 (STAT3). In addition, a PD-L2-dependent mechanism inhibits the function of $CD8^+$ T cells via mast cells, suppressing antitumour immunity in OS.

To our knowledge, for the first time, we elucidate a tumorigenic role for mast cells with immunosuppressive properties in OS. In addition to increasing with tumour progression, OS-infiltrating mast cells exhibit a negative correlation with patient survival, suggesting that targeting these cells may be a therapeutic strategy for OS.

2. Materials and methods

2.1. Osteosarcoma and adjacent non-tumor specimens

Fresh osteosarcoma tissue (OST) (necrosis-free, homogeneous cellularity) and adjacent non-tumor tissue (ANT) a minimum of 5 cm away from the tumour were obtained from patients who underwent surgical resection at the Chongqing Traditional Chinese Medicine Hospital, People's Hospital of Chongqing Hechuan and Chongqing Hechuan Traditional Chinese Medicine Hospital (The number of patients was 54). In all patients, no chemotherapy or radiation had been administered before the sample was collected. Tumours were classified according to TNM by the Union for International Cancer Control (UICC) (8th edition). Ethics approval was obtained from the Chongqing Hospital of Traditional Chinese Medicine's Ethics Committee, and written informed consent was obtained from each subject.

2.2. Differentiation and culture of primary human umbilical cord blood-derived mast cells

Human umbilical cord blood obtained from the Department of Obstetrics and Gynaecology was used to differentiate and culture human umbilical cord blood-derived mast cells (hCBMCs). To isolate $CD133^+$ cells from cord blood, Ficoll-Paque Plus was used for density gradient centrifugation, and CD133 microbeads were used for purification. StemSpan serum-free expansion medium supplemented with penicillin/streptomycin, human recombinant (hr) stem cell factor (SCF), hr IL-6, and hr IL-3 was used for the culture of $CD133^+$ cells for the first 6 weeks. A 10 % solution of foetal calf serum (FCS) was added to the culture medium beginning at week 6, and the culture medium was used until week 10 for experiments.

2.3. Preparation of single-cell suspensions and flow cytometric detection of the extent of mast cell infiltration

To prepare the samples, the tissue was washed three times with Hank's solution, cut into pieces (3 mm^3) using forceps and ophthalmic scissors, incubated in RPMI 1640 containing 1 mg/ml collagenase IV and 10 mg/ml deoxyribonuclease I and homogenised with Miltenyi Biotech's gentle MACS Dissociator. Further incubation of the cell suspension at 37 °C was performed for 1 h under continuous rotation, and the suspension was filtered through a cell filter (70 μm). The mast cells from autologous OSTs and ANTs were stained with anti-CD45, anti-CD117 and anti-FcεRI antibodies for 0.5 h, and infiltration was detected by fluorescence-activated cell sorting (FACS) (BD Biosciences).

2.4. Flow cytometry detection of Ki-67, chemokine receptors and immunosuppressive molecules on mast cells

As mentioned above, chemokine receptors (CCR2, CCR4, CCR5, CXCR1, CXCR2, CXCR4 and CXCR7) and immunosuppressive molecules (BTLA, CTLA4, galectin-3, ICOSL, PD-L1, PD-L2 and TIM3) on mast cells were detected by multicolour flow cytometry according to standard protocols. Perm/Wash solution was used to fix and permeabilize the cells prior to intranuclear staining. The data were analyzed with FlowJo software (TreeStar).

2.5. Preparation of OSCS and ANCS and supernatant-conditioned mast cells

To prepare osteosarcoma culture supernatants (OSCS) or adjacent non-tumor culture supernatants (ANCS), autologous osteosarcomas or non-tumor tissues were plated in 1 ml of RPMI 1640 medium for 24 h. After centrifugation, the supernatant was collected. Supernatant-conditioned mast cells were generated by harvesting and culturing human umbilical cord blood-derived cultured mast cells (hCBMCs) in autologous 50 % OSCS or ANCS for 24 h, followed by three washes with RPMI 1640 medium.

2.6. Chemotaxis assay

Tumour-infiltrating mast cells (1×10^5) were isolated from OS through fluorescence-activated cell sorting, and these cells were plated into the upper chambers to crossover 8-μm pore Transwell plates (Corning). The lower chambers were filled with autologous 50 % OSCS or ANCS as chemoattractants. The number of cells that adhered to the bottom of the membrane and those in the lower chambers after 24 h of culture at 37 °C was used to determine migration. Some mast cell suspensions were incubated for 2 h with a blocking antibody against CXCR2 (20 μg/ml, IgG2A) or a control IgG2A. Additionally, some assays included the addition of OSCS containing neutralizing antibodies against CXCL6 (20 μg/ml, IgG1) or control antibodies. A blank and a positive control were placed in the lower chambers by using RPMI 1640 medium and CXCL6 (100 ng/ml), respectively.

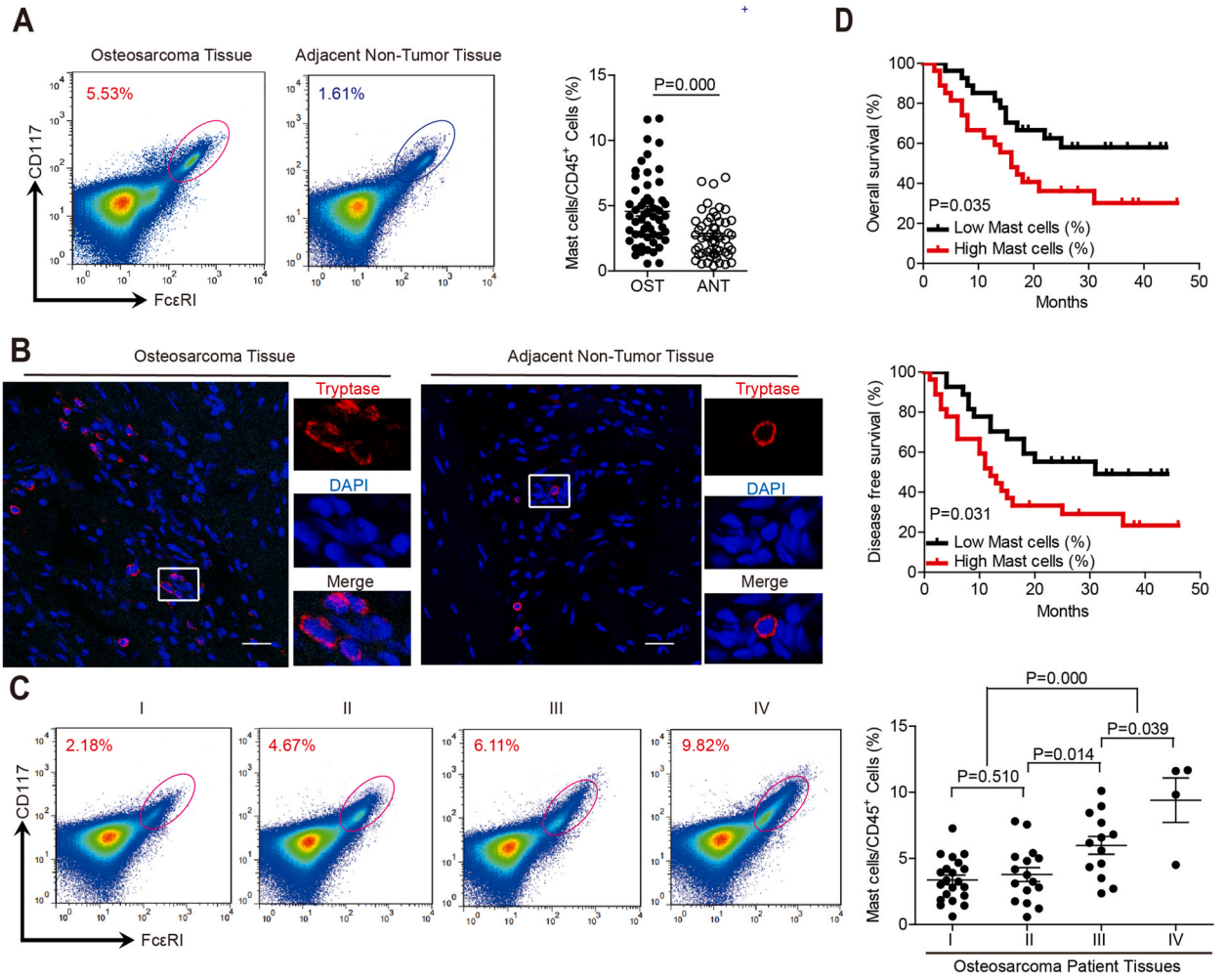

Fig. 1. Mast cells accumulate in OS with disease progression and predict poor patient survival. (A) Representative analysis and Cumulative results of mast cell ($CD45^+$ $CD117^+$ $Fc\varepsilon RI^+$ cell) gating on $CD45^+$ cells in OST and ANT. (B) Representative analysis of $tryptase^+$ (red) mast cell distributions in OST and ANT by immunofluorescence staining. Scale bars: 100 μm(C)Dot plots of surface molecule staining for mast cells gating in $CD45^+$ cells among TNM stages with OS. Cumulative results from 54 OS patients were shown. (D)Kaplan-Meier plots for overall survival and disease-free survival by median mast cell percentage (4.185 %). The horizontal bars in panels A and C represent mean values. Each ring or dot in panels A and C represents 1 patient. OST, osteosarcoma tissues and ANT, adjacent non-tumor tissues. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.7. Mast cell stimulation

The hCBMCs were stimulated with 50 % OSCS and 50 % autologous ANCS, 50 % OSCS at various time points (such as 3, 6 or 12 h), or different concentrations of OSCS (10 %, 20 %, 50 %) for 12 h. In addition, a neutralizing antibody against human G-CSF (20 μg/ml) or multiple different human recombinant (hr) cytokines (100 ng/ml) was used to stimulate hCBMCs for 12 h with 50 % OSCS. After stimulation, the cells were harvested for analysis by flow cytometry.

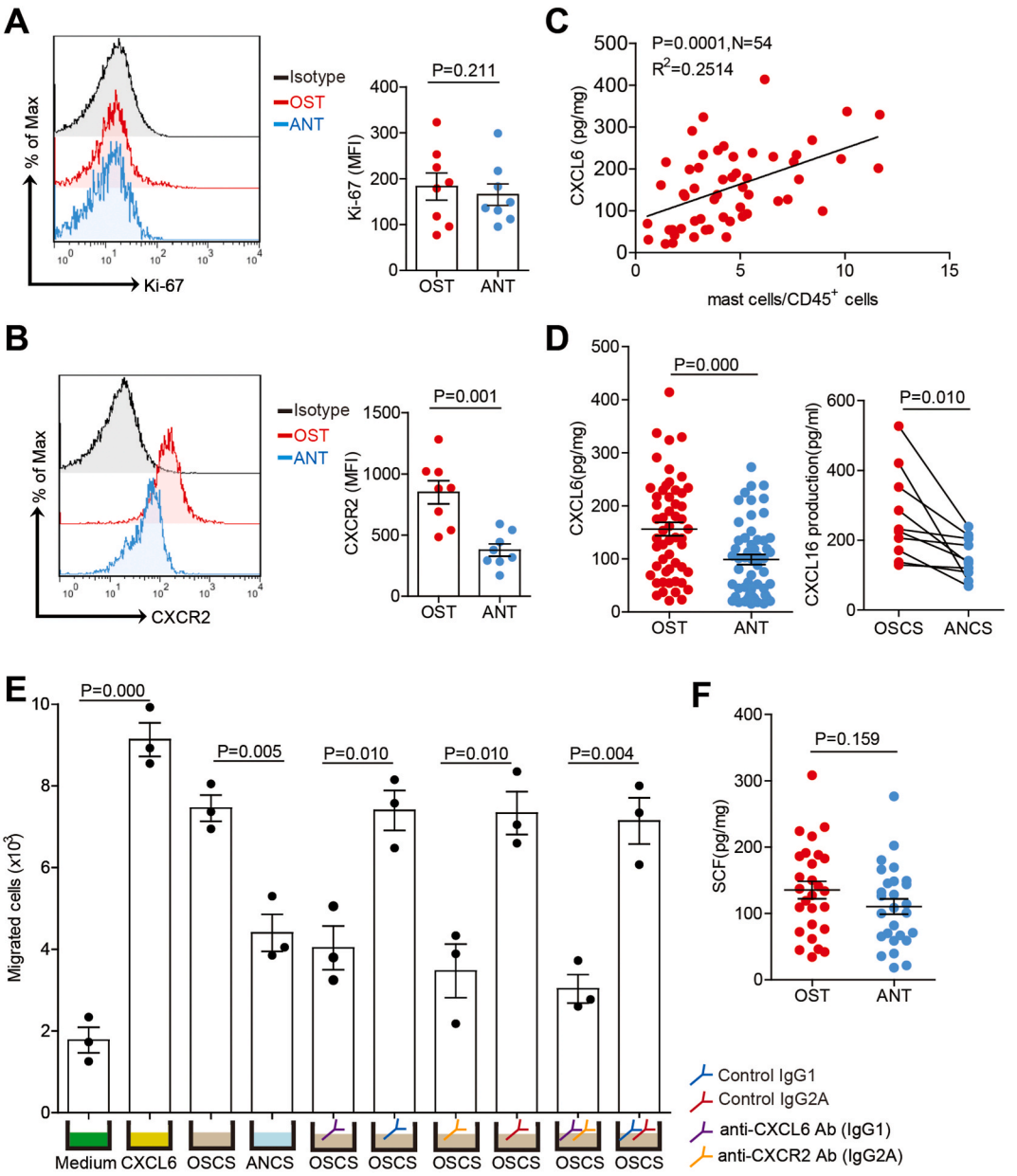

Fig. 2. Chemotaxis mediated by CXCL6-CXCR2 increases mast cell recruitment in OS. (A) OST and ANT mast cells stained for Ki-67. Red and blue histograms show Ki-67 staining respectively, while black represents isotype control. (B) Mast cell expression of CXCR2 molecule in OST and ANT. Red and blue histograms show CXCR2 staining respectively, while black represents isotype control. (C) Based on percentages of mast cells in $CD45^+$ cells and CXCL6 concentrations in OST, a correlation between mast cells and CXCL6 was analyzed in OS. (D) CXCL6 concentration between autologous OST and ANT (n = 54) or between autologous OSCS and ANCS (n = 10) was analyzed. (E) Migration of tumor-infiltrating mast cells was assessed by transwell assay as described in Materials and methods and statistically analyzed (n = 3). (F) SCF concentration between autologous OST and ANT (n = 27). The horizontal bars in panels D and F represent mean values. Each dot in panels C, D, E and F represents 1 patient. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.8. Mast cell-CD8$^+$ T-cell coculture

Magnetic bead-purified peripheral CD8$^+$ T cells (2×10^5 cells/well, 200 μl/well) labeled with carboxyfluorescein succinimidyl ester (CFSE) and autologous mast cells isolated from OST or ANT were incubated in RPMI 1640 medium supplemented with 10 % foetal bovine serum with or without PD-L2 neutralizing antibody (20 μg/ml) at a 2:1 ratio for five days. An alternative coculture method included CFSE-labeled magnetic bead-purified peripheral CD8$^+$ T cells (2×10^5 cells/well, 200 μl/well) at a 2:1 ratio with OSCS- or ANCS-conditioned hCBMCs as described above, with or without a neutralizing antibody against PD-L2 (20 μg/ml). A 96-well plate was precoated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) antibodies, and rh IL-2 (20 IU/ml) was added to the coculture system. Supernatants and cells were harvested after five days of incubation for ELISA and intracellular cytokine staining, respectively.

2.9. Statistical analysis

The results are presented as the mean ± SEM. To analyse the differences between two groups, Student's *t*-test was generally used, but the Mann–Whitney *U* test was employed when variances differed. Overall survival (OS) and disease-free survival (DFS) were defined as the intervals between surgery and death, recurrence, or the last observation, whichever occurred first. As described above, osteosarcoma tissues were collected and single-cell suspensions were prepared, their mast cell infiltration percentages were detected by flow cytometry, and the median of mast cell infiltration percentages was calculated. Patients were divided into mast cell high infiltration and low infiltration groups based on median. Kaplan-Meier curves were plotted using SPSS22.0 based on the survival time (in months) of the follow-up patients as the horizontal coordinate and the cumulative survival rate as the vertical coordinate. Based on Kaplan–Meier survival estimates, we measured survival in months and compared the groups using the log-rank test [14]. Patients who died as a result of unrelated events (e.g., accidental deaths) were excluded. To assess the correlation between parameters, we used Pearson correlation analysis or linear regression analysis, depending on the situation. The Cox proportional hazards model was used for multivariate analysis of prognostic factors for patient survival. All the statistical analyses were conducted using SPSS, and $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Mast cells increase as OS progresses and independently predict poor patient prognosis

To assess the potential role of mast cells in OS, the proportion of mast cells among total CD45$^+$ cells from patient osteosarcoma tissues (OST) and adjacent non-tumor tissues (ANT) was first analyzed. Remarkably, the percentage of mast cells that infiltrated the OST was greater than that in the ANT (Fig. 1A). In addition, mast cell accumulation in the OST was also observed by immunofluorescence staining (Fig. 1B), suggesting a potential role for mast cells in the OS stroma. A significant increase in the number of OST mast cells was also observed as OS progressed, and this increase was most noticeable from stage II onwards (Fig. 1C). Similarly, a correlation was found between the percentage of mast cells in the OST and lymphatic invasion, as well as between tumour stage and size (Supplementary Fig. 1A). We then evaluated the extent of mast cell infiltration in the OST in relation to patient survival. Based on the median level of mast cell infiltration in the tumour tissue, we divided the patients into a high mast cell infiltration group (≥4.185 % median level) and a low infiltration group (<4.185 %). A significant difference was found between patients with high mast cell infiltration and those with low mast cell infiltration at 48 months, and the overall survival and disease-free survival of patients with high mast cell infiltration were lower (Fig. 1D). Overall, these findings suggest that tumour progression and poor survival are associated with intratumoral mast cells in OS patients.

3.2. Chemotaxis mediated by CXCL6-CXCR2 increases mast cell recruitment in OS

As described above, a larger number of mast cells accumulated in the OS microenvironment, leading us to wonder what caused this accumulation. Is the increased infiltration of mast cells in cancer nests caused by *in situ* proliferation? Initially, we determined that mast cells in OS patient tissue barely expressed Ki-67, indicating that those cells were not actively proliferating (Fig. 2A). Therefore, we hypothesized that mast cells might migrate into the tumour microenvironment through chemotaxis. Therefore, we screened the chemokine receptors involved in mast cell migration. The results showed that infiltrating mast cells in osteosarcoma patient tissue did not express CCR2, CCR4, CCR5, CXCR1, CXCR4 or CXCR7 (Supplementary Fig. 2). Nevertheless, mast cells expressed CXCR2 in OS patient tissue, and the expression of CXCR2 on mast cells in OST was higher than that in ANT (Fig. 2B). Interestingly, we also found a positive correlation between the percentage of mast cells among CD45$^+$ cells and the level of CXCL6 (a ligand for CXCR2) in osteosarcoma patient tissue (Fig. 2C). Furthermore, compared with that in ANT and adjacent non-tumor tissue culture supernatants (ANCS), the concentration of CXCL6 in OST and osteosarcoma tissues culture supernatants (OSCS) was significantly greater (Fig. 2D). Through chemotaxis assays, CXCL6-CXCR2 were shown to be functionally important in mast cell recruitment, which demonstrated that OSCS significantly increased the migration of mast cells and that the addition of neutralizing antibodies against CXCL6 and/or CXCR2 to the chemotaxis system weakened the effect of mast cell recruitment (Fig. 2E). SCF is an important regulatory molecule for the differentiation and maturation of mast cells [15], and we rigorously examined its expression level in OST and ANT and found no significant difference, further ruling out that the increased infiltration of mast cells in osteosarcoma was caused by differentiation (Fig. 2F). Based on these findings, the CXCL6-CXCR2 interaction in OS may be responsible for mast cell recruitment to the tumour microenvironment.

3.3. The expression of the immunosuppressive molecule PD-L2 is upregulated on infiltrated mast cells in the osteosarcoma microenvironment

The phenotype of cells determines their function, and in order to better understand the function of these intratumoural mast cells, we performed an immunophenotype study. We screened a variety of mast cell-expressed immunosuppressive molecules and found no difference in their expression levels between the OST and ANT samples (Supplementary Fig. 3). PD-L2 expression on intratumoral mast cells was significantly greater than that on ANT mast cells, suggesting that mast cells may play a key role in OS immunosuppression (Fig. 3A). As a result, we hypothesized that mast cells exhibit immunosuppressive characteristics when located in OS environments. *In vitro*, we mimicked the OS microenvironment with conditioned culture supernatants and stimulated mast cells with OSCS and ANCS, respectively, and surprisingly, OSCS significantly induced the upregulation of PD-L2 expression (Fig. 3B). In addition, we obtained similar results via immunofluorescence staining (Fig. 3C). As predicted, OSCS-conditioned mast cells upregulated PD-L2 expression more significantly than did ANCS-conditioned mast cells in a time- and dose-dependent manner (Fig. 3D and E). These results indicate

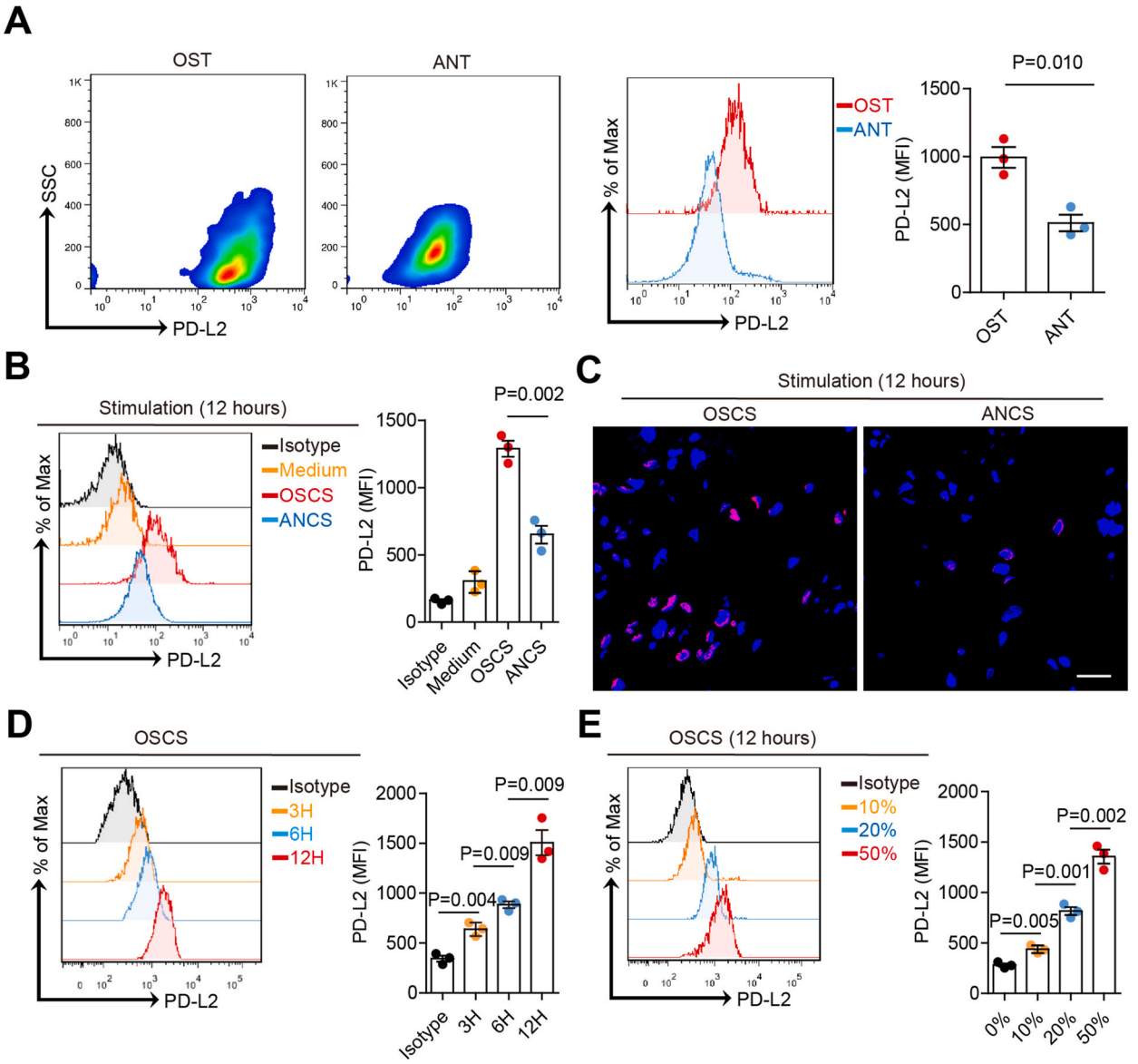

Fig. 3. Expression of the immunosuppressive molecule PD-L2 is upregulated on mast cells infiltrated in osteosarcoma. (A) Representative analysis and statistics analysis of molecule PD-L2 on mast cells in OST and ANT (n = 3). (B) The expression of PD-L2 on hCBMCs after 12 h of exposure to 50 % autologous OSCS and ANCS. (C) HMC-1 cells stimulated by 50 % OSCS and autologous ANCS for 12 h were immunofluorescence stained for PD-L2. Red represents PD-L2, blue represents DAPI-stained nuclei. Scale bar is 50 μm. (D) PD-L2 expression exposed to 50 % OSCS for 3, 6, 12 h. (E) PD-L2 expression exposed to 10 %,20 %, 50 % OSCS for 12 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that PD-L2 expression is increased on infiltrated mast cells in the osteosarcoma microenvironment.

3.4. Mast cell PD-L2 expression is induced by OS-derived G-CSF via the STAT3 pathway

We next explored the mechanism underlying the upregulation of PD-L2 expression on mast cells in the osteosarcoma microenvironment. It has been demonstrated that the tumour microenvironment can contain a wide range of soluble factors, including proinflammatory cytokines [16]. To determine the cytokines that could trigger PD-L2 upregulation on mast cells, we stimulated normal mast cells with cytokines that are known to be present at high levels in tumours, such as IL-1β, IL-6, IL-8, G-CSF, M-CSF, and TGF-β. Our findings revealed that G-CSF alone significantly increased the expression of PD-L2 on mast cells (Supplementary Fig. 4). Furthermore, we found significant increases in G-CSF production in OTSC versus ATCS (Fig. 4A). In addition, a positive correlation was clearly found between PD-L2^{+} mast cell infiltration and the G-CSF concentration in osteosarcoma (Fig. 4B). Then, an antibody that neutralizes G-CSF was added to the coculture system of OTCS-mast cells to assess its possible role in PD-L2 induction. Notably, antibody blockade

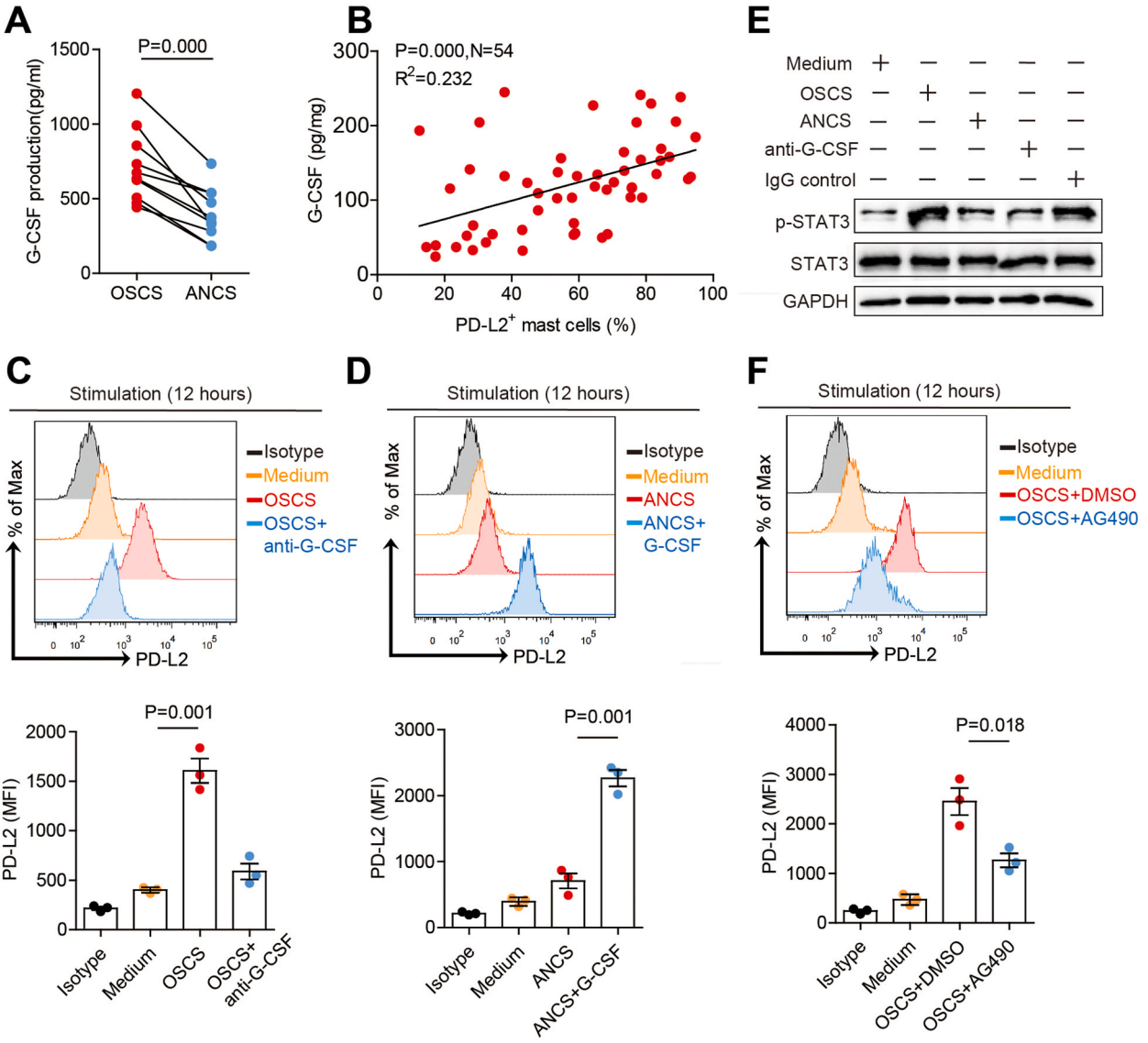

Fig. 4. Mast cell PD-L2 expression is induced by OS-derived G-CSG via STAT3 pathway. (A) G-CSF production between OSCS and autologous ANCS was detected (n = 10). (B) An analysis conducted on the correlation between G-CSF concentration and PD-L2^{+} mast cells in OST. (C) PD-L2 expression on mast cells exposed for 12 h to OSCS and anti-G-CSF antibodies. (D) PD-L2 expression on mast cells exposed for 12 h to ANCS with recombinant G-CSF. (E) Western blots of mast cells treated with OSCS, ANCS, and OSCS with anti-G-CSF antibody or control IgG for 12 h revealed the presence of STAT3 and p-STAT3 (the uncropped version was referred in Supplementary Fig. 6). (F) PD-L2 expression on mast cells exposed for 12 h to OSCS with signaling pathway inhibitor AG490.

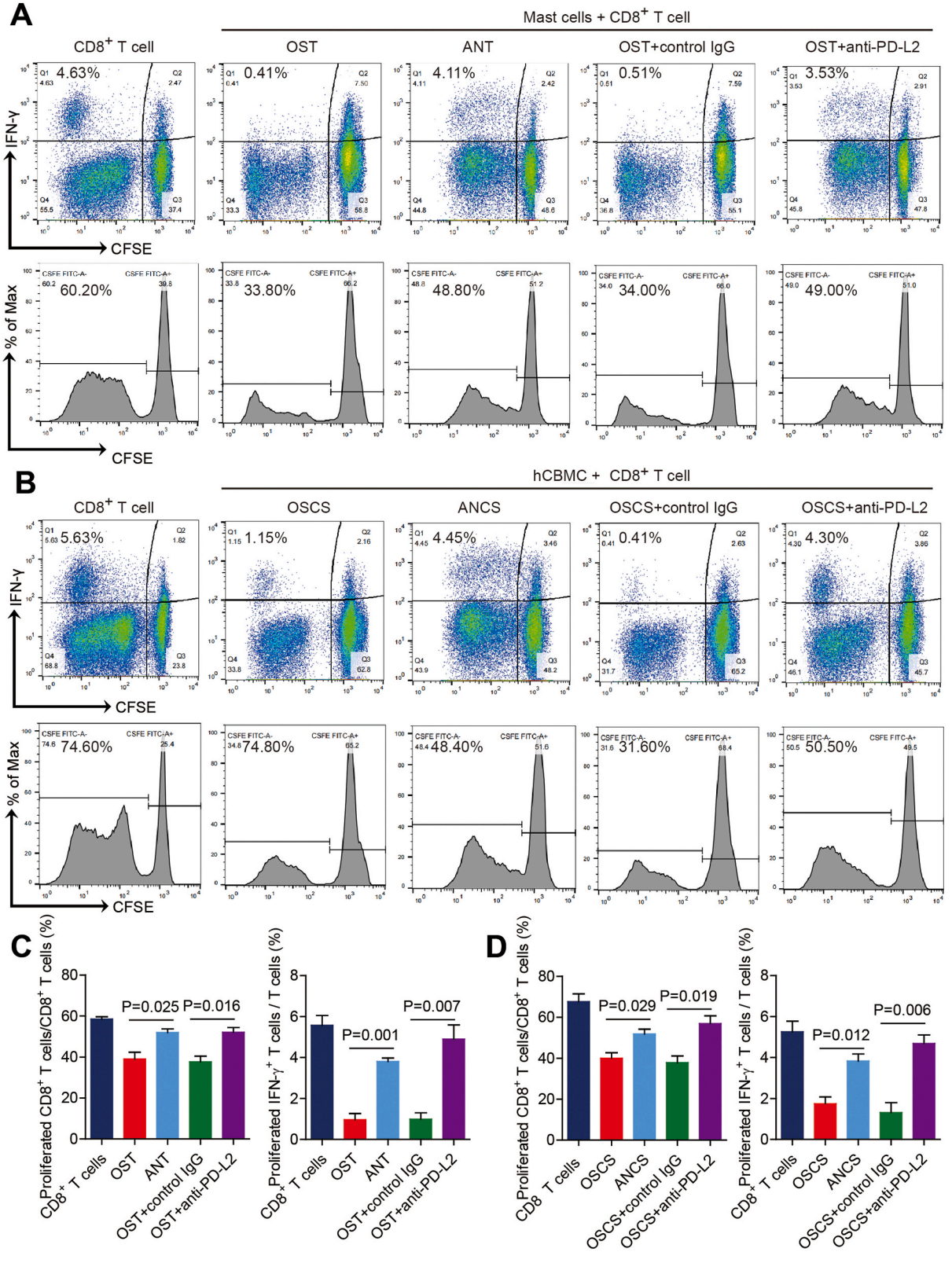

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Fig. 5. Infiltrating mast cells in OS inhibit $CD8^+$ T cell proliferation and antitumour immunity through PD-L2. (A and C) CFSE-labeled OS patients' peripheral $CD8^+$ T cells were co-cultured with autologous mast cells from ANT or OST with anti-PD-L2 antibody or control IgG for 5 days. Representative and statistical analysis of $CD8^+$ T cell proliferation and IFN-γ production were shown (n = 3). (B and D) A co-culture of CFSE-labeled peripheral $CD8^+$ T cells and ANCS or OSCS-conditioned hCBMCs with or without anti-PD-L2 antibody was carried out for 5 days. Representative and statistical analysis of $CD8^+$ T proliferation and IFN-γ production were shown (n = 3).

efficiently inhibited mast cell induction of PD-L2 (Fig. 4C). In addition, we added recombinant G-CSF to ANCS and performed functional recovery experiments, which showed that PD-L2 expression on mast cells was significantly elevated after G-CSF supplementation (Fig. 4D). To investigate which signalling pathways might govern mast cell PD-L2 induction, we pretreated mast cells with the corresponding inhibitors before exposing them to OSCS [17]. In mast cells treated with OSCS, STAT3 (a substrate of the JAK-STAT3 pathway) was predominantly phosphorylated, which could be abolished by the addition of an anti-G-CSF antibody (Fig. 4E). In mast cells treated with OSCS, the inhibitor A490 inhibited the signal transduction of STAT3 to suppress PD-L2 expression (Fig. 4F). These findings show that OS-derived G-CSF might increase mast cell PD-L2 expression by activating the STAT3 pathway.

3.5. *Infiltrating mast cells in osteosarcoma inhibit $CD8^+$ T-cell proliferation and antitumour immunity through PD-L2*

We further investigated the function of infiltrating mast cells in osteosarcoma. The number of mast cells infiltrating tumours was significantly negatively correlated with the number of $CD8^+$ T cells, which suggests that mast cells may impair T-cell immunity and promote tumour progression (Supplementary Fig. 5A). A total of 5 days were spent cultivating autologous peripheral $CD8^+$ T cells and mast cells from patients in the OST and ANT. Coculture studies demonstrated that mast cells infiltrated in osteosarcoma were significantly stronger in inhibiting T-cell proliferation and IFN-γ production than mast cells adjacent to cancer cells, suggesting that tumour-infiltrating mast cells have an immunosuppressive effect on tumour immunity (Fig. 5A and C). In our next objective, we sought to determine the role of PD-L2 in suppressing $CD8^+$ T cells. By incorporating anti-PD-L2 antibodies in a coculture system, we found that blocking PD-L2 attenuated the ability of tumour-infiltrating mast cells to suppress T cells (Fig. 5A and C). It appears that PD-L2 plays an important role in suppressing $CD8^+$ T cells *in vitro* when tumour-infiltrating mast cells are present. Since mast cells in tumour tissues have a stronger inhibitory effect on $CD8^+$ T-cell proliferation and IFN-γ production than mast cells in the paraneoplastic area, we hypothesized that the osteosarcoma microenvironment might play a key role in this process. A 5-day coculture experiment with OSCS- or ANCS-derived primary human umbilical cord blood-derived mast cells (hCBMCs) and peripheral $CD8^+$ T cells was performed to test this hypothesis. As predicted, OSTC-conditioned hCBMCs exhibited greater inhibition of $CD8^+$ T-cell proliferation and IFN-γ secretion (Fig. 5B and D). Then, we tested whether PD-L2 functions in $CD8^+$ T cells and OSCS-conditioned hCBMC coculture systems by adding anti-PD-L2 antibodies. Consistent with our expectations, blocking PD-L2 with antibodies reduced the suppressive effects of OSCS-conditioned hCBMCs on $CD8^+$ T cells (Fig. 5) and increased the production of perforin and granzyme B (Supplementary Fig. 6C). It is clear from these results that mast cells are able to suppress $CD8^+$ T-cell antitumour immunity via PD-L2 in the OS microenvironment.

4. Discussion

Among adolescents, OS is the most common primary bone cancer, with a poor prognosis after metastasis. Despite decades of advancement, OS treatment remains largely confined to surgery and chemo-radiotherapy in the past 30 years. There have been several novel strategies used to treat OS including gene editing, personalized treatment, and molecular targeted therapies (such as monoclonal antibodies, tyrosine kinase inhibitors and angiogenesis inhibitors), however, patients' outcomes have been poor, especially those with more aggressive cancers [18,19]. Consequently, novel treatment strategies are needed in clinical practice, as well as detailed studies on the molecular mechanism that underlies tumorigenesis and metastasis.

Compared with traditional tumour treatments, immunotherapy inhibits OS progression to a greater extent. In OS, the immune microenvironment can be managed to gain a deeper understanding of the mechanisms that contribute to the heterogeneity and progression of the disease. Host adaptive and intrinsic immune cells are important in shaping the tumour microenvironment [20]. There has been considerable research on the roles of adaptive immune cells in OS, but less is known about the roles of innate immune cells [21]. A group of innate immune cells known as mast cells are involved in OS, but the phenotype, regulatory mechanisms and functions of mast cells remain uncertain [22]. In this study, an entirely new type of protumorigenic $PD\text{-}L2^+$ mast cell was identified, and several complementary strategies were employed to elucidate the immunosuppressive phenotypes, mechanism of induction, and biological function of these cells in OS. We found that mast cells contribute positively to the progression of OS. The proportion of mast cells in the tumour increased significantly with increasing OS stage, and increased infiltration of mast cells was negatively correlated with the survival of OS patients, which may be a promising clinical prognostic marker for patients with OS in the future.

Generally, mast cells are involved in allergic reactions [23], but some studies have shown that mast cells in the tumour microenvironment may alter cancer progression and patient outcomes [9,24]. Therefore, it is necessary to investigate the influence of mast cells on OS. Clearly, infiltrating mast cells have a significant impact on OS clinical features, such as distant metastatic spread, stage, and tumour size (Supplementary Fig. 1B). Based on the results of our study, we found a significant increase in the percentage of mast cells in tumours compared to non-tumor tissues. This may be a result of enhanced proliferation or differentiation of mast cells within the tumour or increased migration of mast cells to the tumour tissue. Mast cell Ki-67 expression was rare in the OST, so we excluded the possibility of enhanced proliferation of mast cells in tumour tissue. In addition, SCF is one of the key molecules that induces mast cell differentiation, and the assay revealed that there was no difference in the level of SCF between OST and ANT, suggesting that the

increased number of mast cells in the tumour microenvironment was also not due to increased differentiation. Therefore, it was predicted that mast cells might migrate to the OS microenvironment through chemotaxis. ELISA revealed that the expression level of CXCL6 was greater in OST and OSCS than in ANT and ANCS, which enables the migration of mast cells. In addition, some studies have shown that mast cells also express chemokine receptors in different cancers [25,26]. In the present study, we found that intratumoral mast cells express CXCR2, the receptor for CXCL6, at high levels but rarely express CCR2, CCR4, CCR5, CXCR1, CXCR4 or CXCR7. Thus, we first clarified that the CXCL6-CXCR2 axis mediates mast cell recruitment to the OS micrenvironment. Our study identified a novel mechanism of mast cell chemotaxis in OS.

Mast cell infiltration in tumours has recently been reported [27,28]; unfortunately, the phenotypic characteristics of mast cells and their functions are still poorly understood. Many immune coinhibitory signals are associated with immunosuppression in cancer. One of the main mechanisms underlying the suppression or dysfunction of tumour-specific $CD8^+$ T cells is the cross-talk between PD-1 and PD-L1/PD-L2 [29]. Previous studies have shown that PD-L2 is often expressed in colorectal cancer, cervical cancer and head and neck squamous cell carcinoma [30–32]. To the best of our knowledge, there are no studies showing that PD-L2 expression on mast cells is upregulated in OS. We reported for the first time that PD-L2 was upregulated on infiltrated mast cells in the tumour microenvironment and that these cells had an inhibitory effect on the proliferation of $CD8^+$ T cells and the production of tumour-killing factors (IFN-γ, granzyme B and perforin), which emphasizes the importance of the PD-L2-PD-1 pathway in tumour-associated immunosuppression. Recently, it has been found that the $PD\text{-}L2^+$ neutrophil subset in gastric cancer can also inhibit the tumour-killing effect of T cells by combining PD-L2 with the corresponding ligand PD-1 on T cells, which seems to be consistent with our findings [17].

5. Conclusion

Using the above events as a framework, we identified the mechanisms involved in the progressive immunosuppression within OS (Fig. 6). The recruitment of mast cells to the OS stroma is mediated first by CXCL6-CXCR2 chemotaxis. Then, G-CSF derived from tumours activates STAT3 signalling pathways, which in turn induces mast cells to express PD-L2. Finally, immunosuppressive $PD\text{-}L2^+$ mast cells suppress tumour-specific $CD8^+$ T cells in a PD-L2-dependent manner. It is possible that new therapeutic strategies targeting pathologic $PD\text{-}L2^+$ mast cells may be developed to improve OS in the future.

Declarations

Ethics approval was obtained from the Chongqing Hospital of Traditional Chinese Medicine's Ethics Committee, and the ethics approval number was 2022-ky-74.

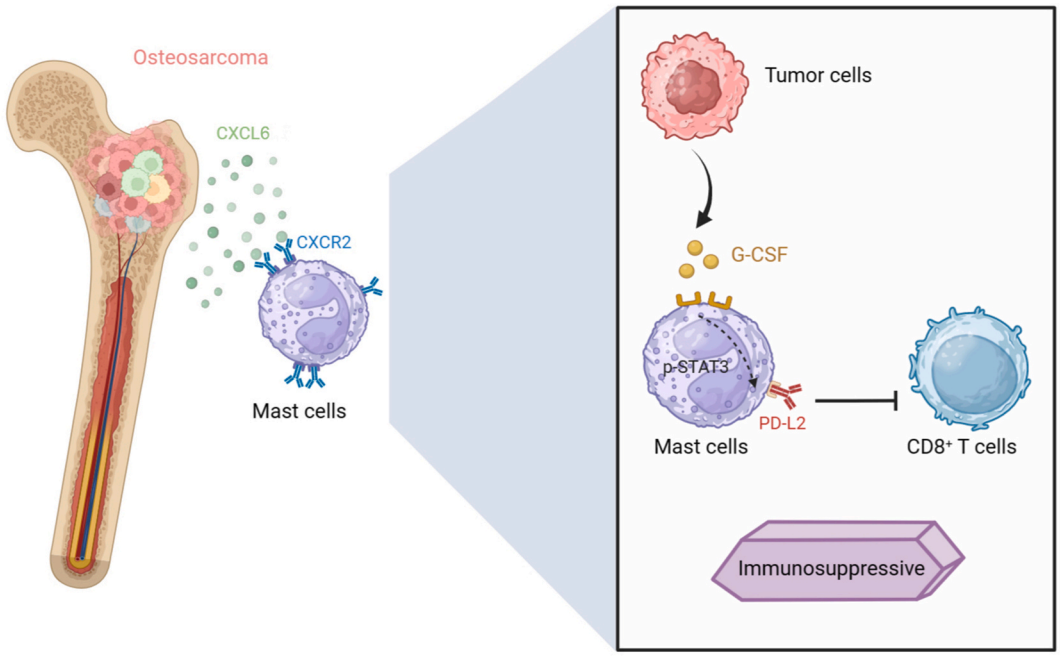

Fig. 6. Model for cross-talks among mast cells, tumor cells, and $CD8^+$ T cells in the OS environment that results in $PD\text{-}L2^+$ mast cell-mediated immunosuppression and tumor progression.

Data availability statement

Data associated with this study have not been deposited into a publicly available repository because all data have been included in this article. The raw data of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Chengguang Wang: Writing – original draft, Investigation, Formal analysis, Data curation. **Zhenbin Lei:** Software, Investigation, Funding acquisition. **Chuanzhi Zhang:** Visualization, Supervision, Resources. **Xiaobo Hu:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34290.

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