Description of the immune microenvironment of chondrosarcoma and contribution to progression

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ABSTRACT
Chondrosarcoma (CHS) is a rare bone malignancy characterized by its resistance to conventional systemic and radiation therapies. Whether immunotherapy targeting immune checkpoints may be active in these tumors remains unknown. To explore the role of the immune system in this tumor, we analyzed the immune environment of chondrosarcomas both in human sample, and in a syngeneic rat model, and tested the contribution of T lymphocytes and macrophages in chondrosarcoma progression. Immunohistochemical stainings were performed on human chondrosarcoma samples and on Swarm rat chondrosarcoma (SRC) model. Selective immunodepletion assays were performed in SRC to evaluate immune population’s involvement in tumor progression.

In human and rat chondrosarcoma, immune infiltrates composed of lymphocytes and macrophages were identified in the peritumoral area. Immune infiltrates composition was found correlated with tumors characteristics and evolution (grade, invasiveness and size). In SRC, selective depletion of T lymphocytes resulted in an accelerated growth rates, whereas depletion of CD163⁺ macrophages slowed down tumor progression. Splenocytes isolated from CHS-bearing SRC showed a specific cytotoxicity directed against chondrosarcoma cells (27%), which significantly decreased in CD3-depleted SRC (11%).

The immune environment contributes to CHS progression in both human and animal models, suggesting that immunomodulatory approaches could be tested in bone chondrosarcoma.

Abbreviations: CD, cluster of differentiation; CDK4, cyclin dependant kinase 4; CHS, chondrosarcoma; HIF, hypoxia inducible factor; IL, interleukin; INF, interferon; NOD2, nucleotid-binding oligomerization domain 2; MMP, matrix metalloproteinase; PD-1, programmed death 1; PD-L1, programmed death ligand 1; SRC, Swarm rat chondrosarcoma; TAM, tumor-infiltrating macrophage; TIL, tumor-infiltrating lymphocyte; TNF, tumor necrosis factor; Treg, T regulator lymphocyte

Introduction
Chondrosarcoma (CHS) is the second most common skeletal malignancy after osteosarcoma, with a worldwide incidence of 1/200,000 per year.¹ CHS is a heterogeneous group of tumors arising preferentially in long bones or in the pelvis and characterized by the capacity of tumor cells to synthetize cartilage. Because of their dense extracellular matrix, low percentage of dividing cells, and poor vascularity, CHS are known to be highly chemo- and radio-resistant.²,³ The only curative treatment for CHS remains surgery with large en bloc tumor resection or amputation to ensure local control of tumor and to prevent future metastases.¹⁴ CHS’s 10-y survival rate ranges from 29% to 83% depending on CHS subtype and grade: Novel therapeutic approaches are therefore urgently needed.¹⁵ Cyclin-dependent kinase 4 (CDK4), matrix metalloproteinases (MMP), SRC, AKT, hypoxia-inducible factor (HIF), mTOR are possible relevant targets in CHS.⁷⁻¹³

Little is known regarding CHS microenvironment, besides its major proteoglycan (PG) contents and its hypoxic nature compared with normal tissues or benign cartilaginous tumors.¹⁴ These characteristics are considered the main limitations of therapeutic agents efficiency in CHS.¹⁵ CHS immunological microenvironment also remains largely unexplored, including its description and its potential involvement in CHS progression. The crosstalk between tumor cells and immune cells has been extensively studied in many tumors over the last 20 y,¹⁵ showing that tumor immune microenvironment is involved in tumor control and progression. Indeed, CD8⁺ T cells and pro-inflammatory M1 macrophages play in most cancers an antitumoral role, whereas Treg and M2 macrophages may promote tumor progression in most cancers,¹⁷,¹⁸ with the possible exception of metastatic osteosarcoma.¹⁹

To date, the analyses of the immune environment of bone sarcoma have led to the following conclusion. For osteosarcoma,
the presence of tumor-infiltrating lymphocytes (TIL) is of good prognosis and the presence of M2 tumor-associated macrophages (TAM) is associated with metastases suppression.

In this study, we report on the description and role of immune infiltrates in CHS. CD8+ T cells and CD163+ cells were observed at the margins of CHS and their presence was found associated with tumor's progression.

Results

Chondrosarcoma immune microenvironment and tumor progression

The localization, lineage and density of immune cells present in CHS microenvironment were analyzed in a cohort of 26 grades I–III conventional CHS.

CD4+, CD8+ T cells and CD163+ cells (monocytes/macrophages) were observed in all the 26 tumors and were mostly located in the peripheral area of the tumor (Fig. 1A). Density of lymphocytes and macrophages varied among patients and ranged from minor to dense (Table S1). On each tumor, positive cells for each immune marker were counted on five different areas located at the margin of cartilaginous nodules and surrounding muscles (magnification 80×) (Fig. 1A). The highest density of T cells was observed in invasive grade II and in grade III CHS.

Immune infiltrate density was correlated with tumor aggressiveness. Indeed, patients with above the median of CD8+, CD4+ and CD3+ immune infiltrates density had a better overall survival than patients with lower peritumoral CD8+, CD4+ and CD3+ counts (p = 0.04), (Fig. 1B) and tended to have better PFS (p > 0.05). An inverse correlation between CD8+ T cells count and tumor size was observed (Fig. 2A).

Figure 1. Immune infiltrate in human chondrosarcoma. (A) From top to bottom representative IHC staining of CD8+, CD3+, CD4+ and CD163+ cells (black arrow) in human chondrosarcoma (original magnification 80×). The interface between the cartilaginous nodules and the fibrous zone is delaminated by a red line. For each figure, the inset shows a representative cell staining (magnification 400×). (B) Immune populations are correlated with patients’ survival. CHS patients OS was compared in two groups based on the median of the immune cell counts. (C) Immune populations and CHS relapse. Patients were split in two groups based on the median of immune cell counts.
The density of CD163+ cells ranged from negligible to important (Table S1); the highest density of CD163+ cells was found in grade III CHS (Table S1). A correlation between high CD163+ cell count and tumor size was observed (Fig. 2A). CD163+ infiltrations did not correlate with overall survival and relapse (Fig. 1B and C).

CD8+ and CD163+ were found inversely correlated in this series of 26 tumors ($R^2 = 0.2, p = 0.035$) (Fig. 2C). The CD8+/CD163 ratio, i.e., the number of CD8+ T cells over M2 related macrophages count is inversely correlated with tumor size (Fig. 2B) and tumor invasion (Fig. 2D). The third grade of CHS was separated into four groups depending on the invasion statuses of the tumor (Table S2). CD8+/CD163 ratio, correlated with tumor group: the less aggressive tumors had the lowest CD8+/CD163 ratio, whereas G4 and G3 infiltrating tumors had the highest CD8+/CD163 ratio. The same observation was made with CD8+ infiltration, whereas the opposite effect was found with the CD163+ infiltration (Fig. 2D).

**SRC immune microenvironment recapitulates human chondrosarcoma**

Immune infiltrates were then analyzed on rat CHS from 17 untreated progressive SRC. As for human CHS, CD3+, CD8+ T cells and CD163+ cells (monocytes/macrophages) were detected at the periphery of all tumors (Fig. 3A). On each tumor, positive cells for each immune marker were counted on five different areas located at the margin of cartilaginous nodules and surrounding muscles (magnification 80×) (Fig. 3A). In the SRC model, the CD8+/CD163 ratio and the CD8+ count were inversely correlated with tumor size (Fig. 3B and C). On the opposite, CD163+ count was correlated with tumor size (Fig. 3C).

**T lymphocytes and macrophages in chondrosarcoma progression**

To further investigate the involvement of each immune population in CHS progression, selective depletions of T lymphocytes or macrophages were performed in SRC model.

Macrophages were targeted using either liposome clodronate (which deplete macrophages population) or mifamurtide (which enable the conversion from pro-tumoral M2 macrophage to pro-inflammatory M1 macrophage). T-cell depletion was obtained using azathioprine. In animals treated by liposome clodronate or mifamurtide, a lower infiltration of CD163+ cells in tumor microenvironment was observed.
azathioprine treated animals, CD8⁺ and CD3⁺ T cells were significantly decreased in tumor’s environment (Fig. 4A).

Both liposome clodronate and mifamurtide affected tumor progression. Animals treated with these agents had significantly smaller tumors compared with control animals (respectively 1,127 ± 177 mm³ and 1,294 ± 195 mm³ versus 1913 ± 202 mm³, p < 0.05 on day 18) (Fig. 4B). In rats treated with liposome clodronate or mifamurtide, the tumor growth rate was lower than in the control group: tumor proliferation rate was respectively 16.23 ± 2.19 in the liposome clodronate treated group and 24.32 ± 3.42 in the mifamurtide treated rats versus 63.17 ± 14.32 in the control group at day 18 (p < 0.005) (Fig. 4C). Both agents significantly delayed tumor progression and time necessary to reach the maximum tumor volume (2,500 mm³) (respectively 26.38 ± 1.65 d and 22.71 ± 1.98 d versus 16.92 ± 0.70 d in the control group, p < 0.05) (Fig. 4D). At day 34, three animals remained alive in the liposome clodronate and mifamurtide treated groups (Fig. 4D).

The opposite effect was observed when lymphocytes were depleted using azathioprine. In this case, tumor growth rate was increased (91.90 ± 21.48 for azathioprine-treated group versus 46.33 ± 7.36 for the control group, p < 0.05 at day 14) (Fig. 4C) with a significantly higher tumor volume than the control groups (respectively 2,349 ± 86 mm³ versus 1,434 ± 169 mm³ at day 14, p < 0.0005) (Fig. 4B). Azathioprine-treated rats reached the maximum tumor volume (2,500 mm³) sooner than the control group (12.33 ± 0.41 d versus 16.92 ± 0.70 d, p < 0.0005) (Fig. 4D). Fourteen days after initiation of the treatments all animals from the azathioprine-treated group were dead, whereas 10 animals remained alive in the control group (Fig. 4D).

These two sets of data suggest that macrophages play a protumoral action, whereas lymphocytes repress CHS growth possibly by specific cytotoxic activity directed against CHS cells.

To elucidate this point, cytotoxicity of splenocytes from different treatment groups was analyzed and production of pro-inflammatory cytokines was measured in rat sera.

The cytotoxic activity of splenocytes from progressive SRC (n = 6), azathioprine treated SRC (n = 6), mifamurtide treated SRC (n = 8), liposome clodronate-treated SRCs (n = 6) and healthy animals (n = 6) was evaluated against primary CHS cells isolated from SRC tumors.

Splenocytes isolated from healthy animals did not have a cytotoxic activity against CHS cells (E:T ratio of 100:1, specific lysis of 2.5 ± 1.7%). Conversely, CHS cells were lysed by splenocytes isolated from CHS-bearing rats: (27.0 ± 6.5% was obtained at the E:T ratio 100:1). The splenocytes from azathioprine, treated rats had a lower cytotoxic activity on CHS cells. (11.3 ± 3.5% at the same E:T ratio of 100:1 (p < 0.0005). Splenocytes isolated from treatment targeting macrophages had a non-significantly lower cytotoxic activity (p > 0.05) (Fig. 5A). These results confirm the hypothesis that effectors CD8⁺ T cells are located in CHS microenvironment and play an antitumoral role and maintain CHS growth. Two key cytokines INFγ and TNF-α were analyzed on all animal sera. INFγ and TNF-α were detectable in the sera of all CHS animals while undetectable in healthy animals (Fig. 5B–D). The three modulatory agents used (azathioprine, liposome clodronate and mifamurtide) increased the level of seric INFγ and TNF-α in the sera of all CHS animals while undetectable in healthy animals (Fig. 5B–D). None of the immunomodulatory treatments caused changes in TNF-α level (Fig. 5C and D).

**Discussion**

CHS is a rare and heterogeneous family of mesenchymal tumors, accounting for 20% of bone malignancies, whose treatment results have remained largely unchanged over the last three decades. The phenotypic features of this tumor, i.e., a
dense extracellular matrix, a low percentage of dividing cells, and a poor vascularity, contribute to CHS chemo- and radio-resistance.1,21 The aim of this study was to describe the immune cell infiltrates associated with CHS, and determine their functional role in CHS progression, first in human samples and then a syngeneic rat model. Indeed, CHS is an unfrequent tumor, thus collecting enough non-decalcified FFPE embedded samples to constitute a cohort is quite challenging. Despite these limitations, we collected 26 samples from grades I–III conventional CHS. Nevertheless, to conduct pertinent analyses in CHS, a reliable animal model is required. For this purpose we conducted our study both in human CHS samples and in a relevant animal model recapitulating the clinical evolution of conventional CHS.

Immune infiltrate composed of lymphocytes and macrophages was found located in the peritumoral area both on human CHS samples and in the rat CHS model (SRC). A high density of CD163+ macrophages was associated with a more invasive tumor, whereas a high CD8+ tumor infiltrate was associated with less aggressive tumors in both models as well. This is noteworthy, considering that the SRC tumors are isogenic in these animals, suggesting that the parameters governing infiltration are not only related to the genomic alteration of the tumor. Similar results were observed in malignant pleural mesothelioma where high amounts of CD163+ and low count of CD8+ cells were associated with local tumor outgrowth.22 Of interest CD8+ T-cells infiltration is higher in low grade non-invasive CHS, whereas CD163+ macrophages infiltration is higher in high-grade invasive tumors. These observations seem to indicate that CHS can modulate its immune environment to promote the invasion in the soft tissues. Also, like melanoma CHS’s grade/aggressiveness seem to be linked to tumor

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**Figure 4.** Effects of selective depletion on tumor’s immune environment. (A) Average immune cells infiltration (CD3+ , CD8+ and CD163+) in each treatment groups at the end of the experimentation. Azathioprine caused a decrease in CD3 and CD8+ cells, whereas mifamurtide and liposome clodronate affected CD163+ cells. Counts were made on five areas per tumor (200 × magnification). (B) Tumor growth evolution in control and CD163 (mifamurtide or liposome clodronate treated) or CD3/CD8+ (azathioprine treated) depleted chondrosarcoma. (C) Tumor’s evolution expressed as relative tumor size in control and CD163 or CD3/CD8+ (azathioprine treated) depleted chondrosarcoma. (D) SRC survival in control and CD163 (mifamurtide or liposome clodronate treated) or CD3/CD8+ (azathioprine treated) depleted chondrosarcoma (*p < 0.05; **p < 0.005).
immunogenicity: the less aggressive tumors being less immunogenic and being less infiltrated by immune effector cells.23

In our cohort of 26 conventional CHS, patients with infiltrates above the median for CD4\(^+\), CD8\(^+\) and CD3\(^+\) counts had a better overall survival and were correlated with smallest tumors. The presence of CD8\(^+\), CD4\(^+\), or CD3 lymphocytes at the periphery of the tumor is of good prognosis in human CHS. Similar observations, on the role of lymphocytes population, have been reported in other cancers\(^{16}\) among which melanoma\(^{24}\) and osteosarcoma.\(^{20}\)

No significant correlation was found between CD163\(^+\) infiltration and overall survival or relapse of CHS patient and was associated with biggest tumors. In high-grade metastatic osteosarcoma, TAM (both M1 and M2 subtypes) are associated with a good prognostic in patients who respond to chemotherapy, whereas in carcinomas TAM are often considered to be protumoral and are associated with bad prognosis.\(^{25}\) We also observed an inverse correlation between CD163\(^+\) TAM and CD8\(^+\) lymphocytes infiltration and show that the presence of TAM could be a barrier to CD8\(^+\) infiltration in the tumors. Such results have also been reported in gastric carcinoma.\(^{26}\)

These observations conducted in 26 conventional CHS patients need to be validated on a larger cohort.

CHS is a rare tumor and biological materials available to study the implication of immune populations in this pathology are scarce. A relevant animal model is necessary to address this question. Given the similar features of SRC with the human counterpart, this model is well suited for such studies; unlike in most sarcoma models established in immunocompromised animals,\(^ {27,28}\) the tumors are established on immunocompetent animals, and grow orthotopically.

The SRC model mimics human grade II CHS in terms of growth and therapeutic resistance.\(^ {29}\) Analyses of SRC showed that SRC immune microenvironment also mimics its human counterpart. In this model, CD8\(^+\) T cells and CD163\(^+\) macrophages infiltrations were correlated with tumor aggressiveness: smaller tumors had a high CD8\(^+\) T-cells count and low CD163\(^+\) infiltrate, whereas larger tumors were characterized by low CD8\(^+\) T cells and high CD163\(^+\) infiltrates. These results confirmed that CHS’s immunological microenvironment plays a role in the tumor progression, the CD8\(^+\) T cells repressing tumor progression and CD163\(^+\) cells having a pro-tumoral action. Both cell components act independently since the depletion of each population in the SRC model affects tumor growth.

Immune infiltrates can affect CHS progression by two mechanisms of action. CD8\(^+\) T cells can directly kill CHS cells. As no infiltration of immune cells in the cartilaginous nodules was found, this cytotoxic action would take place the peritumoral area where immune effectors can encounter tumor cells. Immune infiltrates can also affect CHS progression through the secretion of soluble factors and cytokines. Indeed, it was already shown in osteosarcoma that macrophage-stimulated pro-inflammatory signals could secrete soluble mediators that inhibit tumor cell growth and that immune cells located in the bone environment produce cytokines that modulate osteosarcoma growth.\(^ {30,31}\) Such an effect could be expected in CHS as proinflammatory cytokines are found in CHS.

Azathioprine, an agent that blocks the proliferation of T cells and prevents the differentiation of naïve and memory T cells in effectors T cells,\(^ {32}\) was used to perform T-cell depletion in SRC. Azathioprine affected tumour’s immunological microenvironment causing a net decrease in T cells associated with faster growing tumors and a dramatic decrease in animal’s survival. Splenocytes from progressive SRC have a specific cytotoxic action directed against CHS cells that is absent in azathioprine treated rats, indicating that T cells play an antitumoral role in this CHS model, as in other tumor models.\(^ {16}\)

M2 TAMs\(^ {33,34}\) have been shown to present a protumoral activity.\(^ {18,35}\) Liposome clodronate, which induces macrophage’s death by apoptosis,\(^ {36}\) caused a significant decrease in CD163\(^+\) cells associated with a significantly increased survival of the animals\((p<0.05).\) Mifamurtide, a ligand of NOD2, which drives M2 to M1 macrophages conversion,\(^ {30}\) reduced the density of CD163\(^+\) cells in CHS microenvironment and resulted in an increase in animal’s survival, confirming that phagocytic immune cells and mainly CD163\(^+\) macrophages may play a role in CHS progression, and can be modulated to induce tumor regression. Indeed, in high grade metastatic osteosarcoma the conversion of M2 to M1 macrophages induced by chemotherapy was found to increase patients survival.\(^ {19}\) These data associated to the fact that mifamurtide has already shown its potential therapeutic value in osteosarcoma\(^ {37}\) clearly

Figure 5. Cytotoxic and inflammatory responses in chondrosarcoma. (A) Specific cytotoxicity of splenocytes isolated from tumor-bearing animals. Nontumor-bearing animals did not show a specific cytotoxic activity directed against chondrosarcoma cells. This activity was present only in chondrosarcoma-bearing rats and was decreased by selective depletion of CD163 or CD3/CD8\(^+\) cells ($^{\ast\AST}p < 0.0005$). (B) Serum IFN$\gamma$ levels in normal animals, progressive chondrosarcoma CD163 or CD3/CD8\(^+\) depleted chondrosarcoma-bearing rats ($p < 0.05$). (C) Serum TNF-$\alpha$ level in normal animals, progressive chondrosarcoma CD163 or CD3/CD8\(^+\) depleted chondrosarcoma-bearing rats. Data are given as the mean of each marker measured $\pm$ SE. All values are in pg/mL. (D) Serum levels of INF$\gamma$, TNF-$\alpha$ in normal animals, progressive chondrosarcoma, CD163 or CD3/CD8\(^+\) depleted chondrosarcoma-bearing rats. Data are given as the mean of each marker measured $\pm$ SE. All values are in pg/mL ($N = 7$).
indicate that modulating macrophages populations by favoring M2 to M1 conversion could be a therapeutic approach for CHS.

In metastatic osteosarcoma model, blockade of PD-1/PD-L1 showed potent antitumor action. Due to the role of the immune microenvironment that we observed in CHS, the blockade of this pathway could be interesting in the pathology. Yet, a recently published paper shows no expression of PD-L1 in conventional CHS. Despite this, it is known that PD-L1 expression assessed by IHC can vary depending on the antibody used, staining method and the stage of the pathology as it was seen in NSCLC. Given IHC high variability, it seems to us that the use of PD-L1 IHC staining to select patients that could benefit from anti-PD-L1 antibody therapy should be avoided and replaced by another more reliable technic like genomics analysis.

In conclusion, this work shows that CHS is infiltrated with CD8+ lymphocytes and CD163+ M2 macrophages in the marginal zones of the cartilaginous nodules, both in human and SRC model. We also show that the infiltration of those two populations is correlated with the presentation, and outcome of the disease. The contribution of lymphocytes and macrophages to the tumor progression was demonstrated respectively by azathioprine and liposome clodronate/mifamurtide in the SRC model. This study opens the path to the possibility to use immunotherapy approach for CHS, like blocking immune checkpoint (i.e., PD1/PD-L1, CTLA4) or immunomodulation.

Materials and methods

Cell cultures

The human bone marrow (K-562) and natural killer (NK-92) cell lines were all obtained from the ATCC (LGC Standards) and were cultured at 37 °C, under 5% CO2, using DMEM or RPMI medium (for NK92 cell line) supplemented with 10% FBS, 2% L-glutamin, and penicillin/streptomycin solution (10,000 U/mL; 10,000 μg/mL) (GIBCO ThermoFisher Scientific, Waltham, USA). Primary rat CHS cells and chondrocytes were isolated from SRC or healthy animals by collagenase digestion and cultured in DMEM medium supplemented with 10% FBS, 2% L-glutamine, and penicillin/streptomycin solution (10,000 U/mL; 10,000 μg/mL).

Animal protocols

Experiments were conducted in accordance with the European and French laws and were validated by the local animal ethical evaluation committee C2EA-UCBL55, protocol number: DR2014-55. Animals were maintained and experiments performed in the pathogen-free animal facility "SCAR" at the Rockefeller Medicine faculty (Agreement # A 69 388 10 01).

The transplantable orthotopic SRC model has been previously described. This model is a grade II CHS with mild cellular atypia. It mimics its human counterpart in terms of aggressivity and chemo-resistance. Tumors were grafted on 25-d-old Sprague-Dawley rats (Charles River Laboratories, L’Arbresle, France). Briefly, tumor fragments (10 mm3) were transplanted on the right posterior tibia of the rats after periostal abrasion. Upon establishment of palpable tumors, i.e., 10 d after tumor implantation, animals were randomly divided into treatments groups: PBS (1 mL; n = 14) azathioprine (2 mg/kg; n = 14; Sigma-Aldrich, St-Louis, USA), mifamurtide (0.1 mg/kg; n = 14; Adooq Bioscience, Irvine, USA), liposome clodronate (50 mg/kg; n = 14; ClodronateLiposomes.com, Amsterdam, The Netherlands). Treatments were administered IP 5 d a week for azathioprine and twice a week for the two other groups. Rats were treated over a period of 5 weeks or till tumors reached 2,500 mm3. Tumor growth was monitored by regular visual inspection and tumor dimensions were measured every 2–3 d. Tumor volume was calculated using the formula: Volume = (longest tumor diameter × (shortest tumor diameter)2)/2. At the completion of treatment, rats were sacrificed, and tumors and spleens were harvested. Part of tumors and spleen were used for CHS cells isolation and splenocytes harvesting, whereas the remaining samples were fixed in 10% formalin for further analyses. Blood was collected by cardiac puncture and allowed to clot at room temperature to obtain serum for ELISA analysis.

Splenocytes harvesting

Spleens were mashed on 40-μm-cells strainer (BD Biosciences, San Jose, USA) placed on top of a 50 mL Falcon tube, using a syringe plunger. The collected cells were centrifuged (250g, 5 min, 4°C) after addition of 10 mL RPMI medium supplemented with 10% FBS. To remove red blood cells, pellet was incubated 3 min at room temperature in ACK red blood cell lysis buffer (GIBCO) before being centrifuged (250g, 5 min) and resuspended in complete RPMI medium.

Cytotoxicity assay

A nonradioactive, fluorometric cytotoxicity assay using calcein–ace-toxymethyl (Calcein-AM; Calcein-AM; ThermoFisher C3099) was performed to measure the lysis of rat CHS cells and chondrocytes (further named target cells) by splenocytes. Target cells (1 × 106/mL) were labeled with Calcein-AM (10 μmol/L) at 37°C for 1 h before being washed twice in complete culture medium and plated at 105 cells/wells in round bottom 96-well microtiter plates (Nunc, P8116, Sigma-Aldrich, St-Louis, USA). Target cells where then incubated with 400 μmol/L Sulfinpyrazone (Sigma-Aldrich) in complete medium (1 h, 37°C) to avoid spontaneous calcein release.

Splenocytes isolated from animals of all treatment groups were used as effector cells and added to targets cells at E:T ratios ranging from 100:1 to 12.5:1. Effectors and targets were incubated 6 h at 37°C under normal culture conditions. Targets cells spontaneous and maximum release were assessed respectively by plating target cells alone in 96 wells or by incubating target cells in presence of 2% of Triton X-100 for 6 h under normal culture conditions.

Supernatants were then collected (100 μL) by centrifugation (250g; 5 min) and transferred in transparent 96 wells flat bottom plates (Nunc, P7366, Sigma-Aldrich). NK-92 and K-562 cells lines were used as positive control. For each sample, calcein release was measured on Tecan Infinite F500 (Tecan Group Ltd., Switzerland) (excitation filter: 485 ± 9 nm; bandpass filter: 530 ± 9 nm). Specific lysis induced by the effectors
was calculated according to the formula:

\[
\text{Specific sample lysis} = \left(\frac{(\text{maximal release} - \text{spontaneous release})}{(\text{sample release} - \text{spontaneous release})}\right) \times 100.
\]

All tests were run in triplicate.

**Measurement of cytokines secretion**

Levels of seric IL-10, IL-1β, TNF-α and IFNγ from animals of the different treatments groups were determined by ELISA assays as per the manufacturer’s instructions. IL-1β, TNF-α and INFγ ELISA kits were from Peprotech (Rocky Hill, NJ, USA); IL-10 ELISA kit was from Sigma-Aldrich.

**Phenotypic analyses of chondrosarcoma immune microenvironment**

CHS immune microenvironment was analyzed from 52 SRC and 26 conventional grades I–III human CHS samples treated in our institution between 2004 and 2012. Patients’ CHS grade and infiltration status were analyzed by a pathologist by taking into account radiologic, macroscopic and microscopic observations. For each patient, FFPE included samples was selected from the intramedullary part of the tumor and chosen based on the vascular density observed at the margin of the cartilaginous nodules. Deparaffinization and rehydration were done under standard procedures using xylene (VWR International S.A.S, Fontenay Sous Bois, France) followed by ethanol gradient (VWR).

For SRC, 5-μm sections from formalin-fixed, paraffin-embedded (FFPE) tumor blocks were stained with the following primary antibodies: CD3 (Ventana, Tucson, AZ, USA), CD8+ (1/100), CD163 (1/300),) (respectively OX-8; ED2; AbD SeroTech, Oxford, UK), CD8+ and CD163 primary antibodies were revealed using an unconjugated goat anti-mouse secondary antibody and CD3 by unconjugated goat anti-rabbit antibody (respectively AI-9200, AI-1000; Vector Lab, Burlingame, CA, USA; dilution 1:100) followed by avidin–biotin complex and DAB peroxidase (VECTASTAIN Elite ABC Reagent, ImmPACT reagent; Vector Lab). Counterstaining was performed with hematoxylin (Sigma-Aldrich).

For human CHS samples, 5-μm sections from FFPE blocks were deparaffinized and rehydrated. Immunostaining for CD3, CD8+, CD4+ and CD163 was performed on an automated Ventana Discovery XT staining system. Primary antibodies to CD3 CD8+, CD4+ and CD163 were all from Ventana (respectively clone 2GV6, SP57, SP35, MRQ-26). All primary antibodies were revealed with a biotinylated anti-Rabbit secondary antibody and a DABMap Kit followed by counterstaining with hematoxylin (all reagents from Ventana). For all IHC, human tonsils and rat spleens were used as positive controls. All antibodies negative controls were obtained by omitting relevant primary antibody and incubating directly the slides in presence of secondary antibody.

All slides were analyzed using a Nikon microscope (Eclipse Ni-E Nikon Corp.).

**Quantitative analysis of chondrosarcoma immune infiltrates**

Quantitative analysis of CHS immune infiltrates was similarly conducted on SRC and human CHS slides. CHS’s immune cells were counted in five representative areas with highest density of infiltrates cells located at the periphery of the tumors. Sections were scored based on the intensity and the extent of the staining using the following grading: 1: when 1–25% of positive cells were encountered in the field; 2 when 26–50% positive in the field; 3 when we encounter 50% and more positive cells.

**Statistical analysis**

Tumor growth inhibition (TGI) was defined as the difference between the mean tumor volume (MTV) of the control group and the MTV of the drug-treated group, expressed as percentage of the MTV of the control group at the end of the experiment:

\[
TGI = \left[1 - \left(\frac{\text{MTV}_{\text{drug treated}}}{\text{MTV}_{\text{control}}}\right)\right] \times 100.
\]

All the data are reported as the sample mean ± the standard deviation (SD). Pairwise comparisons between means of different groups were performed using Student’s t-test (two-tailed, unpaired) where, for each couple of normally distributed populations, the null hypotheses that the means are equal were verified. The difference between two subsets of data are considered statistically significant if Student’s t-test gives a significance level (p value < 0.05). Multiple comparisons were performed using univariate analysis of variance (ANOVA).

Kaplan–Meier analysis was performed to estimate the survival probability of different treatment groups at a given time. The median time to endpoint (TTE) and its corresponding 95% confidence interval (CI) were calculated.

All statistical analyses were performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Funding**

This research was supported by The Liddy Shriver Sarcoma Initiative and La Ligue contre le Cancer comité de Saône et Loire.

**Author contributions**

FAS conceived the study, draft the manuscript and performed every experiment. IR and AV helped on in vivo and in vitro experimentation. AVD and JVM performed the chondrosarcoma patient’s selection and evaluated IHC staining. CC, JYB and AD participated in its design and coordination, and critically revised each draft of the manuscript. All authors read and approved the final manuscript.
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