



HAL
open science

The immune landscape of chondrosarcoma - potential for therapeutic targeting of CSFR1+ macrophages

Iseulys Richert, Anne Gomez-Brouchet, Corinne Bouvier, Gonzague Du Bouexic de Pinieux, Marie Karanian, Jean-Yves Blay, Aurélie Dutour

► To cite this version:

Iseulys Richert, Anne Gomez-Brouchet, Corinne Bouvier, Gonzague Du Bouexic de Pinieux, Marie Karanian, et al.. The immune landscape of chondrosarcoma - potential for therapeutic targeting of CSFR1+ macrophages. *Journal of Bone Oncology*, 2020, 20, 10.1016/j.jbo.2019.100271 . hal-03216092

HAL Id: hal-03216092

<https://amu.hal.science/hal-03216092>

Submitted on 3 May 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License



Research Paper

The immune landscape of chondrosarcoma - potential for therapeutic targeting of CSFR1 + macrophages



Iseulys Richert^a, Anne Gomez-Brouchet^b, Corinne Bouvier^c, Gonzague Du Bouexic De Pinieux^d, Marie Karanian^e, Jean-Yves Blay^{a,e}, Aurélie Dutour^{a,*}

^a CRCL/CLB INSERM, Cell Death and Pediatric Cancers Team, U1052, UMR5286, CNRS U5286, 28 rue Laennec, 69373 Lyon cedex 8, France

^b Department of Pathology, IUCT-Oncopole, CHU of Toulouse, Université de Toulouse 3, UMR1037 INSERM, ERL5294 CNRS, Toulouse, France

^c Department of Pathology, APHM La Timone, Aix Marseille University, MMG, France

^d Department of Pathology, Centre Hospitalier Universitaire Tours, 37000 Tours, France

^e Department of Pathology, Centre Léon Bérard, Lyon, France

A B S T R A C T

Survival rate for Chondrosarcoma (CHS) is at a standstill, more effective treatments are urgently needed. Consequently, a better understanding of CHS biology and its immune environment is crucial to identify new prognostic factors and therapeutic targets.

Here, we exhaustively describe the immune landscape of conventional and dedifferentiated CHS. Using IHC and molecular analyses (RT-qPCR), we mapped the expression of immune cell markers (CD3, CD8, CD68, CD163) and immune checkpoints (ICPs) from a cohort of 27 conventional and 49 dedifferentiated CHS. The impact of the density of tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAMs) and immune checkpoints (ICPs) on clinical outcome were analyzed.

We reveal that TAMs are the main immune population in CHS. Focusing on dedifferentiated CHS, we found that immune infiltrate composition is correlated with patient outcome, a high CD68+ /CD8+ ratio being an independent poor prognostic factor ($p < 0.01$), and high CD68+ levels being associated with the presence of metastases at diagnosis ($p < 0.05$). Among the ICPs evaluated, CSF1R, B7H3, SIRPA, TIM3 and LAG3 were expressed at the mRNA level in both CHS subtypes. Furthermore, PDL1 expression was confirmed by IHC exclusively in dedifferentiated CHS (42.6% of the patients) and CSF1R was expressed by TAMs in 89.7% of dedifferentiated CHS (vs 62.9% in conventional).

Our results show that the immune infiltrate of CHS is mainly composed of immunosuppressive actors favoring tumor progression. Our results indicate that dedifferentiated CHS could be eligible for anti-PDL1 therapy and more importantly immunomodulation through CSF1R + macrophages could be a promising therapeutic approach for both CHS subtypes.

1. Introduction

Chondrosarcoma (CHS) accounts for 20% of primary bone tumors [1]. Based on morphological features and clinical evolution, different subtypes of CHS are described [2], the most frequent being conventional CHS (80–85%). Three grades of conventional CHS exist based on cellularity, on the composition of the matrix (chondroid and/or myxoid), on nuclear atypia and on the presence of mitosis. The 10-year

survival rate of these tumors is 85% for grade I CHS and 29% for grade III CHS, and the risk of local and distant recurrence increases with histological grade [3,4]. Among the remaining CHS subtypes, dedifferentiated CHS (10%) is characterized by the occurrence of two components separated by a distinct interface: a well-differentiated cartilaginous tumor (enchondroma, grade I or II CHS) adjacent to a typically high grade sarcoma [5]. Dedifferentiated CHS has a high metastatic potential and presents a worse prognosis than conventional

Abbreviations: APC, Antigen Presenting Cells; B7H3, B7 superfamily member-H3; CD, Cluster of Differentiation; CHS, Chondrosarcoma; CSF1, Colony Stimulating Factor 1; CSF1R, Colony Stimulating Factor 1 Receptor; CTLA4, cytotoxic T-lymphocyte-associated; HH, hedgehog; HLA, Human Leucocytes Antigen; ICOS, Inducible Costimulator; ICOSL, inducible costimulator ligand; ICP, Immune checkpoint; IDH, isocitrate dehydrogenase; LAG3, Lymphocyte activation gene-3; MDR1, Multi Drug Resistance 1; mTOR, mammalian Target Of Rapamycin; PDI, Programmed death 1; PDL1, Programmed Death Ligand 1; SIRPa, Signal regulatory protein alpha; SMO, Smoothed Homolog Precursor; TAMs, Tumor Associated Macrophages; TILs, Tumor Infiltrating Lymphocytes; TIM3, T cell immunoglobulin mucin; Treg, T regulator lymphocytes

* Corresponding author.

E-mail addresses: iseulys.richert@lyon.unicancer.fr (I. Richert), brouchet.anne@chu-toulouse.fr (A. Gomez-Brouchet), Corinne.BOUVIER2@ap-hm.fr (C. Bouvier), gongague.dubouexic@univ-tours.fr (G. Du Bouexic De Pinieux), marie.karanian@lyon.unicancer.fr (M. Karanian), jean-yves.blay@lyon.unicancer.fr (J.-Y. Blay), aurelie.dutour@lyon.unicancer.fr (A. Dutour).

<https://doi.org/10.1016/j.jbo.2019.100271>

Received 27 August 2019; Received in revised form 20 November 2019; Accepted 25 November 2019

Available online 26 November 2019

2212-1374/ © 2019 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

CHS (10-year survival rate of 30%) [6].

Surgical monobloc resection remains the most effective treatment for conventional CHS, systemic treatments (chemotherapy and radiotherapy) having limited efficacy. Over the last 10 years, research has focused on elucidating the biology of chondrosarcoma, with the aim of developing new molecularly targeted therapies. Signaling pathways shown to play a role in CHS, include Hedgehog (Hh), Src, and PI3k-Akt-mTOR [7]. Targeted therapies, such as inhibitors of Hh and Src pathways, have demonstrated meaningful anti-tumor activity in preclinical studies, though the results in early phase clinical studies have fallen short of expectations [7]. It is now recognized that the density and the composition of immune infiltrates in solid tumors are correlated with patient outcome and play a role in tumor progression [8], suggesting that new therapeutic options could include manipulating the tumor microenvironment and its immune infiltrate. Hence, therapeutic approaches aiming at activating immune cells have raised hopes for solid tumor management. However, knowledge on the immune environment and development of immunotherapies for people with bone sarcoma are lacking.

Among bone tumors, the immune infiltrate of osteosarcoma (OsA) is the best characterized. Indeed, tumor-infiltrating lymphocytes (TILs) density and expression of immune checkpoints (ICPs), such as PDL1, are correlated with tumor aggressiveness and patient outcome [9,10]. In addition, previous studies reported the association of a high density of tumor-associated macrophages (TAMs) with better overall survival and suppression of metastasis in high-grade OsA [11,12].

The role of the immune environment in tumor progression is very complex as the balance between pro- and anti-inflammatory effectors is responsible for the activation or inhibition of the anti-tumor immune response.

In addition to immune cells, other actors of the immune environment, such as ICPs are now better described and could be targeted to regain anti-tumor immunity. Among them, (i) B7H3 is expressed by tumor cells and inhibits the immune response [13], (ii) CSF1R, expressed by TAMs, promotes survival and macrophage proliferation [14,15], and (iii) SIRPA, expressed by macrophages, prevents phagocytosis of CD47+ tumor cells [16].

In CHS, few studies have reported the implication of the immune environment in tumor progression [17,18]. One study highlighted the existence of a correlation between immune infiltrate composition, tumor aggressiveness and patient survival in conventional CHS. Authors substantiated their conclusions by analyzing an immunocompetent rodent model in which CD8+ T cells were shown to have an anti-tumoral activity, whereas CD163+ macrophages were pro-tumoral [18]. Another group demonstrated the expression of PDL1 in 50% of dedifferentiated CHS, constituting a potential indication for the administration of anti-PD1/PDL1 antibodies to patients with this tumor subtype [17].

The aim of our current study was to clarify the role of the immune environment in the progression of CHS, more particularly in the dedifferentiated CHS subtype, in order to identify new therapeutic targets. For this purpose, we (1) mapped the immune populations and (2) analyzed the ICPs expressed in a cohort of dedifferentiated CHS and compared its immune landscape to the one of conventional CHS. TILs and TAMs were characterized by IHC, and the expression of ICPs (*OX40/OX40L*, *B7H3*, *TIM3*, *LAG3*, *CTLA4*, *PDL1*, *CSF1/CSF1R*, *ICOS*, *ICOSL*, *CD47*, *SIRPA*) was explored by molecular analyses and the most interesting targets were then validated by IHC. We correlated the composition of the immune landscape with tumor aggressiveness and patient outcome, in order to stratify CHS patients and identify those that might benefit from immunotherapy. Our study emphasizes the immunosuppressive nature of the CHS immune environment and the role of macrophages in high grade CHS immune landscape, highlighting the possibility of using immunomodulation through macrophages as a potential therapeutic target.

2. Material and methods

2.1. CHS cohort

All samples from this retrospective study were handled according to the ethical guidelines for the use of biological material in research described by our institutions.

The patients included in our cohort were diagnosed between 2005 and 2017 in 4 French institutions (CLB, Lyon; IUCT, Toulouse; La Timone Hospital, Marseille; Trousseau Hospital, Tours). The FFPE samples comprised 27 conventional chondrosarcoma (all of which were primary tumors: 3 grade I, 17 grade II and 1 grade III) and 49 dedifferentiated CHS (out of the 49, 7 were biopsies). The dedifferentiated component of dedifferentiated CHS were 36.7% osteosarcoma (16/49 patients), 12.2% undifferentiated spindle cell sarcoma (6/49), 2% RMS (1/49) and 16.3% unknown (8/49). The validated cohort was composed of 29 dedifferentiated CHS for which anonymized clinical information was available (Table 1). IDH1/2 mutational status was known for 7 conventional CHS patients and 12 dedifferentiated CHS patients. The seven CHS patients were all IDH mutated (5 were IDH1 mutated and 2 were IDH2 mutated) and among the 12 dedifferentiated CHS patients 4 were IDH1 mutated and 2 were IDH2 mutated.

All CHS samples were reviewed by experienced pathologists of the GFPO (French Group of Bone Pathologists). None of the patients included had undergone chemotherapy before surgical resection.

2.2. Immunostaining

For each tumor, analyses of immune populations were performed on two 5- μ m thick sections. The immune infiltrates were assessed in 2 non-comitant areas of the tumor section.

To facilitate immunostaining, mainly, the selected FFPE blocks hadn't undergone decalcification, or had been submitted to gentle decalcification (formic acid or EDTA).

Deparaffinization and rehydration were conducted following standard procedures. IHC was performed as described previously [12] on an automated Ventana Discovery XT staining system (Ventana Medical Systems, Innovation Park Drive, Tucson, Arizona 85755 USA, Roche). Immunostaining of T cells was performed with CD3 (clone [2GV6], Ventana) and CD8 (clone [SP57], Ventana) markers. For macrophages, CD68 (clone [KP1], Dako) and CD163 (clone [10D6], Leica) antibodies were used. For PD1 and PDL1 staining, the clones NAT105, (Ventana) and EIL3N (Cell

Table 1

Clinicopathological data of 27 FFPE conventional and 29 dedifferentiated chondrosarcoma of the validated cohort used for survival analyses

Validated cohort	C CHS N = 27	DD CHS N = 29
Gender		
Male	17(63%)	19 (66%)
Female	10(37%)	9 (31%)
Unknown		1 (3%)
Age at diagnosis		
Median	56	57.5
Range	17–80	44–90
Tumor localization		
Extremities	17 (63%)	15 (52%)
Axial and pelvis	10 (37%)	8 (28%)
Unknown		6 (20%)
Tumor size		
< 8 cm	11 (41%)	8 (7%)
> 8 cm	16 (59%)	10 (35%)
Unknown		11 (38%)
Metastatic status		
M–	16 (59%)	11(38%)
M+	8 (30%)	16(55%)
Unknown	3 (1%)	2(7%)

Signaling) were used, respectively. Details and dilutions for each antibody used are presented in the [Supplementary Table 1](#).

CSF1R immunostaining was performed manually. After antigen retrieval (via heat induction in citrate buffer, pH 6.0), the CSF1R primary antibody (clone [SP211], Abcam, 1/100) was incubated overnight at 4 °C. Inactivation of endogenous peroxidases was performed using H₂O₂ (0.3%, 15 min, RT). The primary antibody was detected using biotinylated goat anti-rabbit secondary antibody (AI-1000; Vector lab, Burlingame, CA, USA; dilution 1:100, 1 h, RT) followed by avidin-biotin complex and DAB peroxidase (SK-4100, Vector Lab; dilution 1:300, 30 min, RT). Sections were counterstained in hematoxylin (Vector Lab).

Tonsil and lymph node sections were used as a positive control.

Specimens for which loss of tissue occurred during the staining procedure were not included in the analysis.

2.3. Immunohistochemical evaluation and scoring

Whole sections were evaluated and scored independently by two observers (CB and AGB). Scoring of each marker was reported semi-quantitatively as the percentage of positive cells per total number of tumor cells on the section, irrespective of the staining intensity.

For dedifferentiated CHS, the median expression of each marker (established on basis of the whole cohort) was set as the cutoff value to distinguish between “high” or “low” expression.

Evaluation of the tumor-infiltrating lymphocytes (TILs: CD3, CD8) and tumor-associated macrophages (TAMs: CD68, CD163) was performed by scoring each marker on interpretable slides: 45 dedifferentiated CHS for CD3, 47 for CD8, 47 for CD68 and 49 for CD163 ([Fig. 1A](#)).

Immunoreactivity for PD1, PDL1 and CSF1R was considered positive if detected in more than 1% of cells. Variations in staining intensity of the CSF1R+ cells were scored, and the following criteria were used: - : negative; + : weak but unequivocal staining in some cells; ++ : strong or intense staining.

2.4. Transcriptomic analyses

Expression of the following ICPs: OX40 (*TNFRSF4*), OX40L (*TNFSF4*), TIM3 (*HAVCR2*), LAG3 (*LAG3*), B7H3 (*CD276*), CTLA4 (*CTLA4*), PDL1 (*CD274*), CD47 (*CD47*), SIRPα (*SIRPA*), CSF1 (*CSF1*), CSF1R (*CSF1R*) was analyzed by RT-qPCR on 24 CHS samples (16 conventional and 8 dedifferentiated) and compared to positive and negative controls (MG63 (RRID: CVCL_0426), Saos-2 (RRID: CVCL_0548), Kasumi-1 (CVCL_0589), SW1353 (CVCL_0543) and RD (CVCL_1649)) cell lines described to express high or low levels of these ICPs, according to the Cancer Cell line Encyclopedia GSE36133 [19]. The percentage of tumor cells in CHS samples was evaluated by biobanks hosting these samples, and was estimated at approximately 50%. Frozen samples of dedifferentiated CHS were taken in the dedifferentiated compartment (as confirmed by mirror image sections review).

CHS RNA samples were provided by the Centre Léon BERARD biobank (PGEB) certified AFNOR (NF S 96 900) (Lyon, France) and the Biological Resources Centre of the Assistance Publique Hôpitaux de Marseille, (CRB AP-HM, certified NF S96-900 & ISO 9001 v2015), from the CRB-TBM component (BB-0033-00097).

All cell lines (ATCC, Molsheim, France) were cultured at 37 °C, under 5% CO₂. MG63, SW1353, RD and Saos-2 were cultured in monolayer, with DMEM-Glutamax supplemented with 10% FBS and 1% P/S (Penicillin 10000U/mL; Streptomycin 100,000 µg/mL) (GIBCO, Thermofischer Scientific, Waltham, USA). The Kasumi-1 cell line was maintained in suspension using RPMI-Glutamax supplemented with 20% FBS and 1% P/S. All experiments were performed with mycoplasma-free cells (Mycoclear™, Mycoplasma detection kit, Lonza, Basel, Switzerland). The authentication of cell lines was performed using human Short Tandem Repeat (STR) analysis (ATCC).

RNA from 10⁶ cells was extracted using TRI Reagent® (Sigma Aldrich, St-Louis, USA) according to manufacturer's recommendations.

RNA (500 ng) was then reverse-transcribed using the PrimeScript™ RT reagent Kit (Takara, Bio Europe/Clontech, Saint-Germain-en-Laye, France) according to manufacturer's protocol and diluted at 15 ng/µL in RNase-free water and then stored at -80 °C for further analyses. Quantitative PCR was performed on a LightCycler® 480 Instrument II (Roche, Boulogne-Bilancourt, France), using 2X SYBR® Premix Ex Taq™ (Takara), 1 µM of each primer (Eurofins, Ebersberg, Germany), 2 µL of diluted cDNA and RNase-free water (qsp 10 µL). Amplification conditions were as follows: 5 min at 95 °C followed by 40 PCR cycles (15 s at 95 °C, 30 s at 60 °C). Relative gene expression was normalized against two internal controls, *GAPDH* and *RPLP0* and calculated using the 2^{-ΔΔCT} method. ICP expression in CHS samples is presented as the fold change expression compared to gene expression in the positive control cell line, arbitrarily set at 1.

2.5. Statistical analyses

Clinical data were available for 27 dedifferentiated CHS; survival analyses were performed on this validated cohort. To evaluate, the potential prognostic value of each immune marker, patients of this cohort were stratified into two groups: “high” vs “low” expression of the marker of interest (the cutoff being the median expression of each marker in the whole cohort). All data are reported as the mean ± standard deviation.

All survival rates were estimated using the Kaplan–Meier method with 95% confidence intervals (CI). Overall survival and metastases-free survival were defined as the time from CHS diagnosis to death of any cause, metastasis detection or last follow-up (event censored). Multivariate analyses were performed using the Cox proportional hazard model including age, gender, metastatic status, and the CD68/CD8 ratio in the infiltrate was calculated using Rstudio (R Studio software, Boston, USA, <https://www.rstudio.com/>).

The nonparametric Mann–Whitney test was used to compare mRNA expression levels between control cell lines and tumors using GraphPad prism version 6.00 (GraphPad software, La Jolla, CA, USA, www.graphpad.com). A *p* value < 0.05 was considered statistically significant.

3. Results

3.1. Macrophages are the main population encountered in CHS immune infiltrates

Due to its small size, the conventional CHS cohort served as a comparison cohort for the characterization of the dedifferentiated CHS immune landscape. The immune infiltrate composed of TILs (CD3+, CD8+ cells) and TAMs (CD68+, CD163+ cells) was limited to the peri-tumoral area of conventional CHS, while immune cells were found at the periphery of the tumor but also intermingled with tumor cells in dedifferentiated areas of dedifferentiated CHS ([Supplementary Fig. 1](#)). In both CHS subtypes, TAMs appear to be the most abundant immune population. Indeed, these were primarily constituted of CD163+ TAMs in conventional CHS (median of expression: 25%), whereas TILs were scarce (CD3 and CD8 median of expression: 10% and 8%).

A similar composition of immune infiltrate was observed in dedifferentiated CHS, in which the median percentage of CD68+ and CD163+ cells were 20% and 50%, while it was of 15% for CD3 and 5% for CD8 T cells ([Fig. 1A](#)).

For each immune marker, patients were segregated based on the level of its expression, the cutoff value being the median percentage of positive cells ([Fig. 1A](#)).

In the validated cohort, namely patients for which we had clinical information (*n* = 29), a low expression of CD8 was encountered in 25%

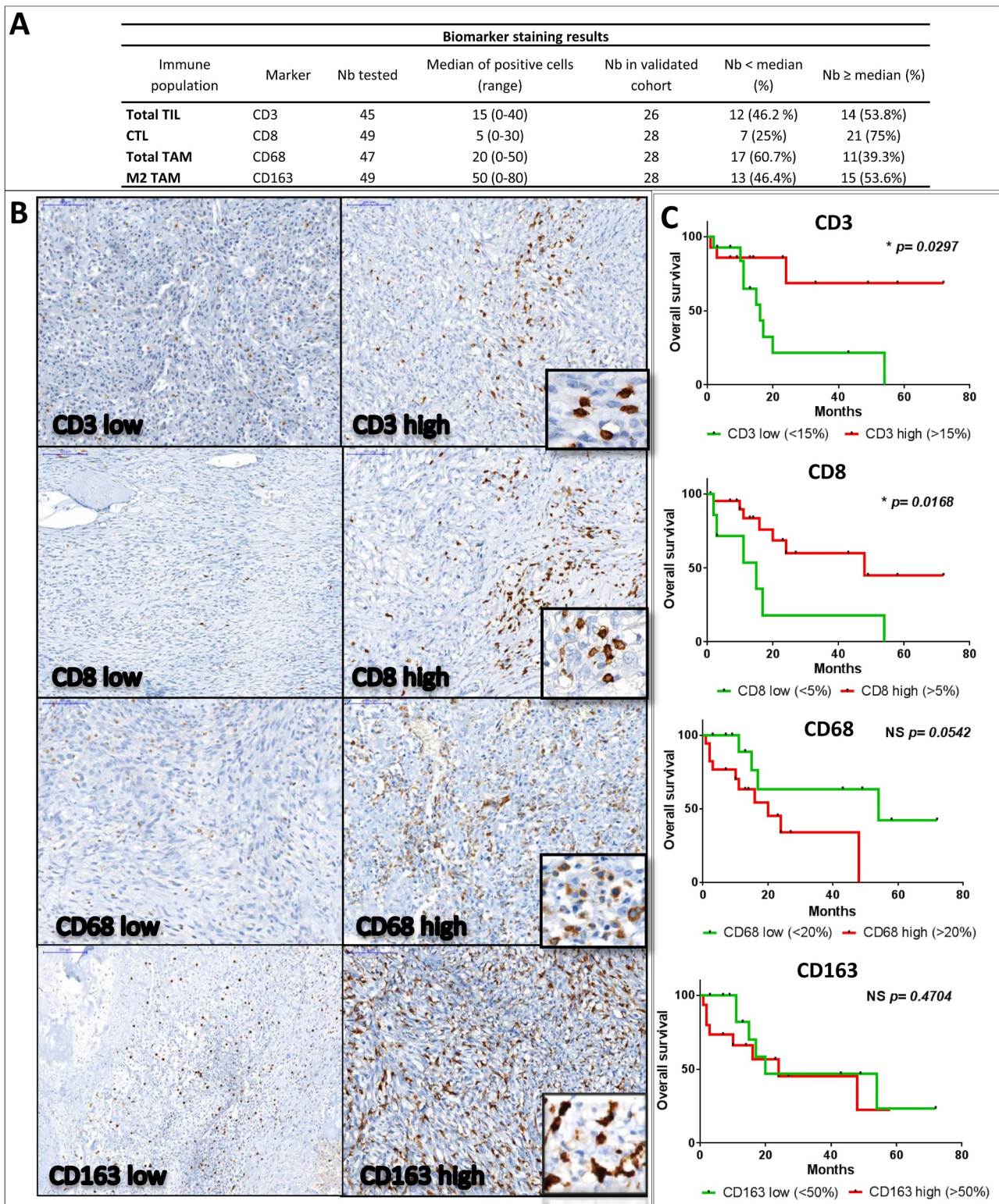


Fig. 1. TIL and TAM infiltrates in DD CHS are prognostic factors. **A.** Biomarker staining results. **B.** Representative images of primary dedifferentiated CHS with low and high CD3 (cut off: 15%), CD8 (cut off 5%), CD68 (cut off 20%) and CD163 (cut off 50%) infiltrates (magnification X200). Frames correspond to the high-power field of each picture (magnification X400). **C.** Kaplan Maier survival analyses according to CD3, CD8, CD68 and CD163 infiltration status. Patients were divided into two groups depending on immune marker expression (high or low), the cutoff value being the median expression of each immune marker. A high CD3 and CD8 infiltrate was associated with better survival ($p < 0.05$), whereas a high CD68/CD8 infiltrate was associated with a poorer survival ($p < 0.005$). (* $p < 0.05$; ** $p < 0.005$). TIL tumor infiltrating lymphocytes; CTL cytotoxic lymphocytes; TAM tumor associated macrophages.

of dedifferentiated CHS, while a high expression was found in 75% of these tumors. For TAMs, a low CD68 expression was found in 39.3% of patients and high expression in 60.7% (Fig. 1A)

Representative staining of low or high density of TILs (CD3, CD8) and TAMs (CD68, CD163) are presented in Fig. 1B.

3.2. The composition of the immune infiltrate of dedifferentiated CHS is correlated with patient survival

We then used the validated cohort, to assess the prognostic value of the immune infiltrate of dedifferentiated CHS. We evaluated whether TIL and TAM densities impact dedifferentiated CHS progression. The Infiltration of TILs (CD3, CD8) and TAMs (CD68, CD163) were not correlated with the clinical features of patients (age, gender, tumor localization) (Supplementary Table 2) Survival analyses based on immune cell infiltrate composition showed that a high density of CD3+ TILs and CD8+ cytotoxic T cells was associated with better overall survival (Fig. 1C; $p = 0.0297$ median survival of 16 months (CD3 low) vs the non-measurable median survival (CD3 high), $p = 0.0168$, median survival of 15 months (CD8 low) vs 48 months (CD8 high)).

Inversely, a high ratio of CD68+ TAMs was associated with poor overall survival (Fig. 1C; $p = 0.0542$, median survival of 20 months (CD68 high) vs 54 months (CD68 low)). More importantly, an elevated CD68/CD8 ratio (> 2) was of poorer prognosis than CD68 density alone (Fig. 2A; $p = 0.0073$, median survival of 16 months (CD68/CD8 high) vs 54 months (CD68/CD8 low)), implying a potential relationship between CD68+ TAMs and CD8+ TILs. In a multivariate Cox regression model including age, gender and metastatic status, a high CD68/CD8 ratio was confirmed to be an independent poor prognostic factor of overall survival (HR = 6.17, $p = 0.00973$) (Fig. 2B).

In dedifferentiated CHS, we evaluated whether immune cell density of the dedifferentiated compartment varies depending on its histological structure. Interestingly, dedifferentiated CHS presenting an osteosarcoma compartment had a higher density of CD68+ TAM (77.8% vs 31.8% in other dedifferentiated subtypes) and a higher CD68/CD8 ratio (72.2% vs 22.7% for other type of differentiation) (Fig. 3A). Survival analyses based on the histological subtypes of dedifferentiated compartment revealed that a high density of CD68+ TAMs tended to be associated with poor survival exclusively in the dedifferentiated CHS presenting a dedifferentiated osteosarcoma compartment (NS, $p = 0.06$, Fig. 3B).

We were unable to assess the relationship between IDH mutation status and immune infiltrate composition as IDH mutation status was known for only 12 patients in dedifferentiated CHS.

3.3. TAM density was associated with metastatic dissemination

We then determined whether a link between the composition of the

primary tumor immune infiltrate and the metastatic potential of the tumor existed. A high CD68+ TAM density was associated with poor MPFS (metastasis progression-free progression survival) (Fig. 4A; $p = 0.049$, median of MPFS 7 months for CD68 high vs 36 months for CD68 low). Moreover, patients with metastases at diagnosis presented higher CD68+ and CD163+ TAM densities than patients with a localized disease (Fig. 4B; $p = 0.026$ and $p = 0.0057$, respectively); 100% of patients with metastasis at diagnosis belonged to the CD68 high and CD163 high groups. Those results indicate that TAMs may participate in the development of metastases.

3.4. Molecular screening of the immune checkpoint landscape of chondrosarcoma

Aside from PD1/PDL1, the expression of other ICPs in CHS has not been reported so far. We thus analyzed the expression of ICPs in these tumors. We screened the expression of a panel of ICPs known to be activators (OX40/OX40L; ICOS/ICOSL) or inhibitors (B7H3, TIM3, LAG3, CSF1/CSF1R, CTLA4, PDL1, CD47/SIRPA) of the immune response. Their expression was first evaluated by RT-qPCR in 16 conventional and 8 dedifferentiated CHS.

Transcriptomic analyses revealed that genes encoding for ICOS, ICOSL, CTLA4, CD47 were not detected in CHS. PDL1 was only expressed in dedifferentiated CHS. The level of OX40 expression decreased with the grade of the tumor: its highest level of expression being found in grade I conventional CHS and the lowest in dedifferentiated CHS (Fig. 5A). CSF1R, B7H3, SIRPA, TIM3 and LAG3 were expressed in both CHS subtypes (Fig. 5A).

We cross-checked the expression of B7H3, PDL1, CSF1, CSF1R and SIRPA in our cohort of conventional and dedifferentiated CHS with tumor cell lines known to express these ICPs at high or low levels (Fig. 5B). This comparison indicated that B7H3 was expressed in both CHS subtypes at the same level as the positive cell line (MG63) (Fig. 5B), while PDL1 was expressed at a similar level as the positive control (Saos2) exclusively in the dedifferentiated subtype (Fig. 5B).

SIRPA, a receptor present on antigen presenting cell (APC) that negatively regulates phagocytosis, was expressed similarly to the positive cell line (SW1353) in conventional CHS, but was weakly expressed in the dedifferentiated subtype (Fig. 5B; $p < 0.05$).

The expression of CSF1, a receptor implicated in macrophage survival and proliferation, differed between the conventional and dedifferentiated CHS, being low in dedifferentiated CHS and as elevated as the control cell line (MG63) in conventional CHS (Fig. 5B). Conversely, the expression of its receptor CSF1R was higher than in the control line in both CHS subtypes suggesting its capacity to respond to CSF1 (Fig. 5B conventional CHS: $p < 0.005$; dedifferentiated CHS: $p < 0.05$).

We then validated previously published data on the expression of PDL1 by both subtypes of CHS reinforced by the analysis of PD1

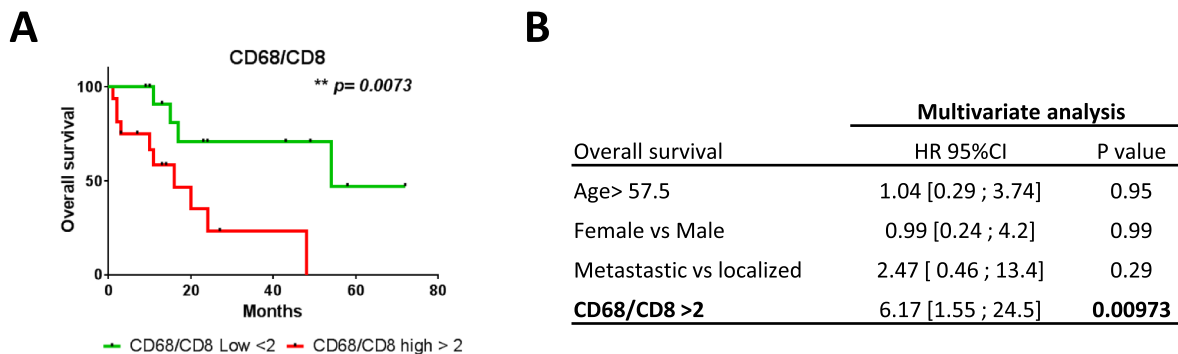


Fig. 2. The CD68/CD8 ratio is a bad prognosis factor in dedifferentiated CHS. A. A high CD68/CD8 ratio is associated with a poorer overall survival (** $p < 0.005$) B. In a multivariate Cox regression model including age, gender and metastatic status, a high CD68/CD8 ratio was confirmed to be an independent bad prognostic factor of overall survival (HR = 6.17, $p = 0.00973$).

A

	Histological subtypes of dedifferentiated compartment		P value
	OsA N=18	Others N=22	
CD3			0,296
<15	10 (55,5%)	9 (40,9%)	
>15	6 (33,3%)	11 (50%)	
CD8			0,9189
<5	6 (33,3%)	7 (31,8%)	
>5	12 (66,7%)	15 (68,2%)	
CD68			0,0025
<20	3 (16,7%)	14 (63,6%)	
>20	14 (77,8%)	7 (31,8%)	
CD163			0,137
<50	6 (33,3%)	12 (54,5%)	
>50	12 (66,7%)	9 (40,9%)	
CD68/CD8			0,0012
<2	4 (22,2%)	16 (72,7%)	
>2	13 (72,2%)	5 (22,7%)	

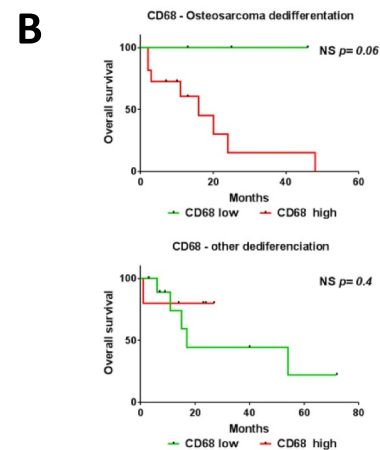


Fig. 3. The histological subtypes of the dedifferentiated compartment is associated with immune cell density and impacts patient survival. A. Dedifferentiated CHS presenting an osteosarcoma compartment with a higher density of CD68 + TAM (77.8% vs 31.8% in other dedifferentiated subtypes) and a higher CD68/CD8 ratio (72.2% vs 22.7% for other type of differentiation). **B.** Survival analyses according to the histological subtypes. We confirmed only for patients presenting an osteosarcoma differentiation that a high density of CD68 + TAMs tended to be associated with poor survival as opposed to other types of differentiation.

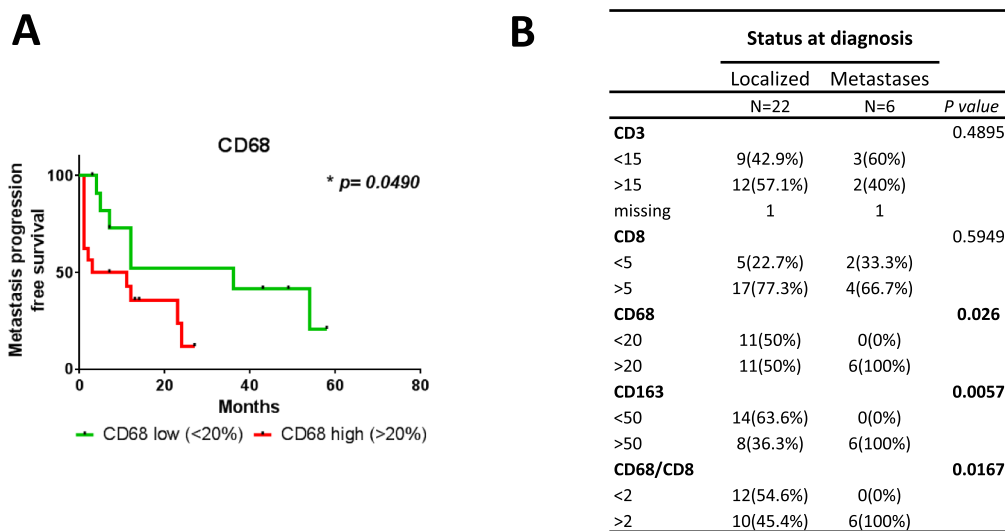


Fig. 4. A high TAM density is associated with the development of metastasis in dedifferentiated CHS. A. Kaplan Maier MPFS (metastasis progression-free progression survival) were established according to CD3, CD8, CD68 and CD163 infiltration. TAM (CD68+) is associated with poorer MPFS (**p* = 0.049). *p* Value obtained by log-rank test. (**p* < 0.05; ***p* < 0.005). **B.** TAM CD68+ and TAM CD163+ density are associated with the presence of metastasis at diagnosis.

expression. In addition, as macrophages are the most abundant immune cells in both CHS subtypes and as CSF1R directed drugs are available, we analyzed the expression of this receptor in CHS.

3.4.1. The immune checkpoints PD1/PDL1 are differentially expressed depending on CHS subtype

First we examined PD1/PDL1 expression by IHC on the whole cohort (27 conventional CHS; 49 dedifferentiated CHS). ICP expression was considered positive when > 1% of PDL1 + tumor cells and > 1% PD1 + lymphocytes were counted in tumor sections (Fig. 6A).

IHC confirmed the exclusive expression of PDL1 in 42.6% (20/49) of dedifferentiated CHS (Fig. 6A)). Of note, PDL1 + tumor cells were exclusively found in the dedifferentiated part of dedifferentiated CHS (Supplementary Fig. 2). As PD1 is expressed by lymphocytes, both conventional and dedifferentiated CHS were positive for this receptor at similar levels: 32% for conventional CHS (8/25); and 28.3% for dedifferentiated CHS (13/46) (Fig. 6A). PD1 + lymphocytes were either adjacent to PDL1 + tumor cells in dedifferentiated CHS or at the periphery in lymphoid aggregates.

ICP expression was not correlated with patient outcome. Indeed, the median survival was similar for patients expressing or not PDL1 (20 months for PDL1 – vs 24 months for PDL1 +) (Fig. 6B). There was no

relationship between PDL1 expression and metastatic status.

3.4.2. CSF1R is highly expressed on macrophages in both CHS subtypes

As TAMs have an important role in CHS progression and CSF1R was highly expressed at the mRNA level, we examined CSF1R expression in CHS by IHC.

CSF1R staining was observed at the membrane of macrophages (Fig. 7A). Indeed, by comparing the staining of CD68 and CSF1R, we validated the similar level of staining of those markers (Fig. 7A)

CSF1R+ macrophages were present at the periphery of both CHS subtypes or in the dedifferentiated compartment of dedifferentiated CHS.

64% of conventional CHS and 84.6% of dedifferentiated CHS were positive for CSF1R (Fig. 7B). Moreover, when considering the intensity of CSF1R staining based on CHS histologic subtype and grading, dedifferentiated CHS presented a higher density of CSF1R+ macrophages (i.e. >20% CSF1R+ TAM in 54% of cases whereas it was of 34 % in conventional CHS) (Fig. 7C).

However, the high density of the different immune cells (CD3+, CD8+, CD68+ and CD163+) was not correlated with the high expression of the different ICPs (PD1/PDL1 and CSF1R).

We analyzed whether CSF1R expression could have a prognostic

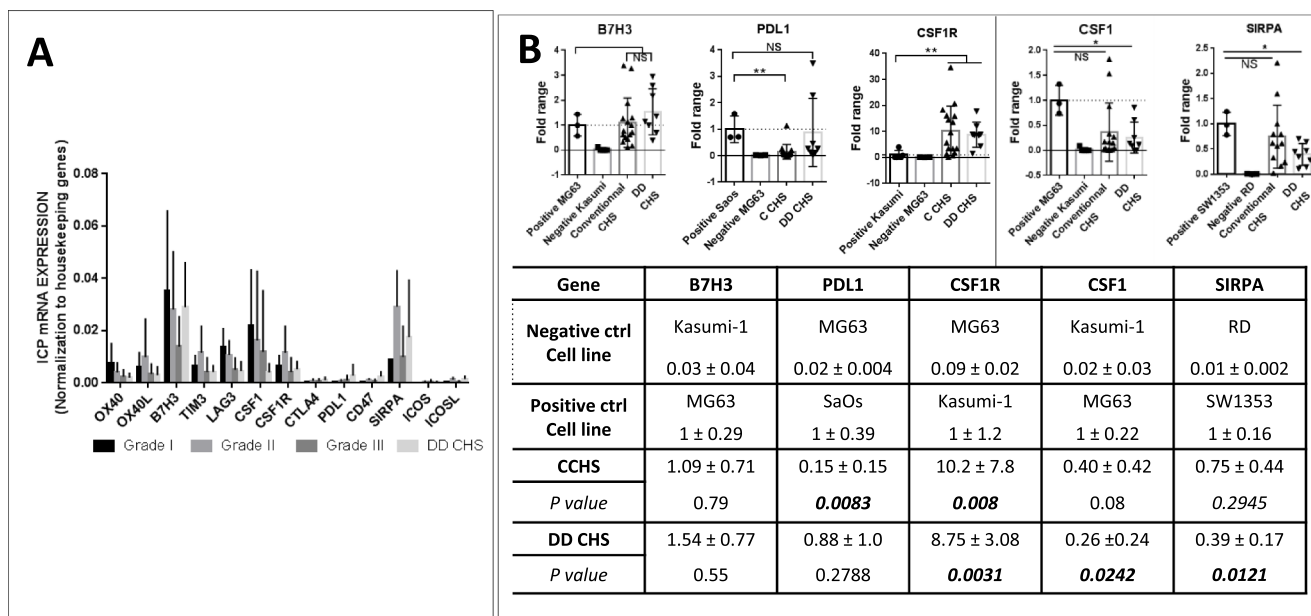


Fig. 5. Immune checkpoint expression in conventional and dedifferentiated CHS. A. Molecular analyses of immune checkpoint (ICP) expression in conventional CHS ($n = 16$) and dedifferentiated CHS ($n = 7$) showed that PDL1 and CTLA4 were expressed at low levels in CHS, while OX40/OX40L, B7H3, CSF1/CSF1R and TIM3 were highly expressed. ICP expression was normalized against housekeeping genes (GADPDH/RPLP0). B. B7H3, PDL1, CSF1, CSF1R and B7H3 expression in CHS. Using positive and negative control cell lines, we evaluated the level of expression of ICP in CHS. This comparison confirmed a high expression of B7H3 (NS) and CSF1R ($p < 0.05$). In both CHS subtypes while PDL1 was only expressed in dedifferentiated CHS (NS). *p* Value was calculated between the positive control and the samples

value for CHS patients. CSF1R expression tended to be correlated with poorer overall survival in dedifferentiated CHS (NS, $p = 0.19$, median survival 20 months for CSF1R+ patients vs 54 months for CSF1R- patients) (Fig. 7D). Inversely, in conventional CHS, the CSF1R

expression was not associated with overall survival (NS, $p = 0.63$). However, the presence of CSF1R+ cells was associated with the metastatic status of dedifferentiated CHS at diagnosis ($*p = 0.02$) (Fig. 7E).

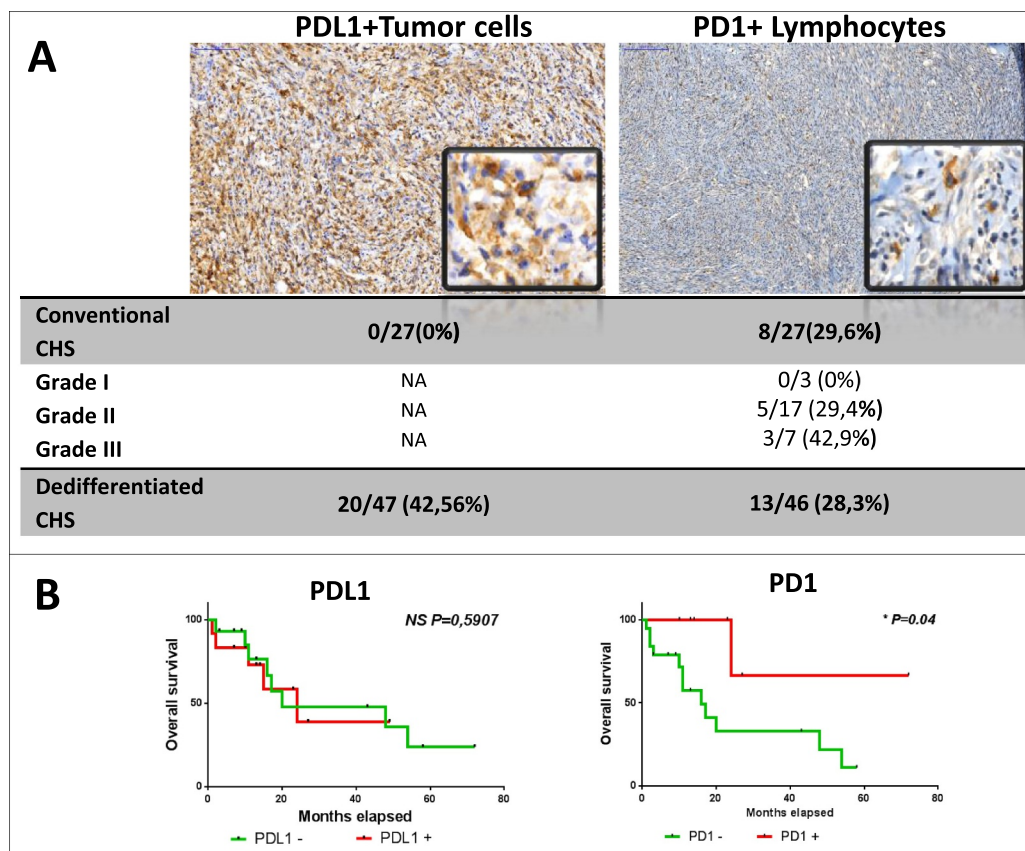


Fig. 6. PD1 and PDL1 expression in conventional and dedifferentiated CHS. PDL1 and PD1 expression analyzed by IHC. Representative images of PDL1+ tumor, PD1+ lymphocytes IHC staining and percentage positivity of those ICPs considering the subtypes and the grade. D, Impact of ICP expression on patient survival in dedifferentiated CHS. PDL1 expression was not associated with overall survival.

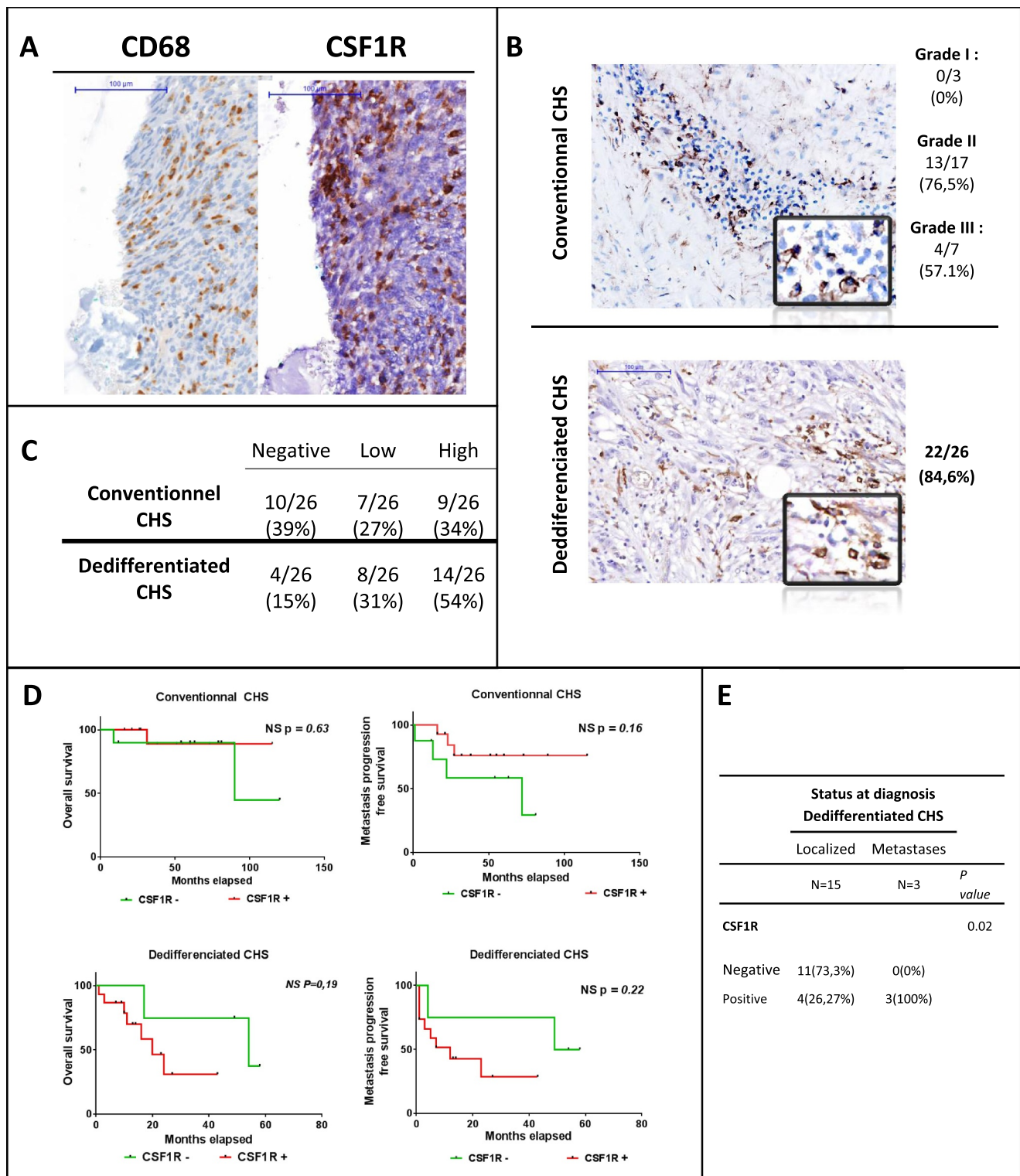


Fig. 7. CSF1R expression in both subtypes of CHS. CSF1R expression was analyzed by IHC. **A.** CSF1R staining was localized at the membrane of macrophages similarly to CD68 staining. **B.** CSF1R positive cases based on CHS subtype and grade. **C.** Percentage distribution of staining density of CSF1R. The percentage of patients expressing a high density of CSF1R+ TAMs was more important in the dedifferentiated subtypes than in the conventional (54% vs 34%). **D.** Prognostic value of CSF1R in CHS The expression of CSF1R was not associated either with overall survival nor MPFS in both subtypes. **E.** CSF1R+ TAMs are associated with the metastatic status at diagnosis in dedifferentiated CHS.

4. Discussion

This study aimed at mapping the immune landscape of CHS to identify which immunotherapy could be applied to patients with these tumors. We particularly focused on dedifferentiated CHS, a rare highly

aggressive CHS subtype with poor prognosis and for which no therapeutic solution exists. Despite the limitations of gathering non-decalcified FFPE samples from this rare bone tumor, we collected 49 dedifferentiated CHS from 4 French reference centers.

In this dedifferentiated cohort, TAMs were the major

immunosuppressive actors of the immune environment of CHS and their density predicted poor survival and development of metastases. We completed the mapping of the immune environment, by analyzing the expression of a large panel of ICPs. We identified other actors of the CHS immunosuppressive environment: PDL1 exclusively expressed by tumor cells in the dedifferentiated CHS and CSF1R expressed by TAMs in both subtypes.

The mapping of the CHS immune environment revealed that TAMs are the main population encountered. We confirmed the exclusive peritumoral location of the immune infiltrates in conventional CHS as reported by Simard et al. [18]. In contrast, abundant immune infiltrate was present in the dedifferentiated areas of dedifferentiated CHS. This infiltrate was mainly composed of CD68+ TAMs. These results are in line with data obtained in bone tumors, such as osteosarcoma, in which TAMs can represent up to 50% of the tumor mass [12].

The abundance of TAMs could be indicative of a role for these cells in tumor progression and of their prognostic value. Survival analyses of dedifferentiated CHS according to the density of TILs and TAMs showed that a high density of CD3+ and CD8+ TILs was predictive of better overall survival while a high density of TAMs (CD68+) was associated with poorer survival. Similar results were observed in other solid tumors (ovarian cancer [20], osteosarcoma [9,8]), in which a high density of TILs was a factor of good prognosis. The presence of TAMs, was associated with poor prognosis in some cancers [21] such as in hepatocellular carcinoma [22,23], gastric cancer [24], esophageal cancer [25], and breast carcinoma [26].

Polarized M1 and M2 macrophages represent the extremes of a continuum of functional states for TAMs. These two macrophage subtypes are characterized by a high plasticity and their ability to alter from one phenotype to the other depending on the cytokines secreted by the tumors or immune cells of the microenvironment.

The classically activated M1 macrophages are potent effector cells that kill tumor cells and produce pro-inflammatory cytokines [21]. M2 macrophages are considered to be pro-tumoral since they block the immune response and promote angiogenesis [21]. Previous studies have indicated that clusters of differentiation CD68 and CD163 are the most common TAM markers [27]. It is worth noting that to distinguish the population, different markers were used: CD68 is a pan-macrophage marker for TAMs and CD163 is the marker commonly used to identify immunosuppressive M2 macrophages. As previously published, we tried to differentiate M1 from M2 macrophages using pSTAT1 and cMAF staining, respectively [12,28]. However new references of these antibodies provided by the supplier did not reliably stain TAMs. Other markers of macrophage functions could be used to discriminate those two phenotypes, iNOS for M1 macrophages and ARG1 for M2 macrophages [29].

Even if CD163 was highly expressed in our dedifferentiated CHS cohort, no correlation between the density of CD163+ TAMs and patient outcome was found. In some sarcomas, the high density of CD163+ TAMs has been correlated with poorer survival [18,30]. Although in osteosarcoma, recent studies converge to indicate that a high CD163+ TAM density is associated with better survival and prevents metastatic spreading [12,31]. Of note, the percentage of CD163+ TAMs was higher than CD68+ TAMs, this unexpected result could be due to the fact that the visual semi-quantitative analyses underestimated the CD68+ TAMs due to the low intensity of the staining. Thus it seems primordial to better characterize these immune cells to ascertain their role.

For this reason, we also considered the balance between the pro-inflammatory and the immunosuppressive actors in dedifferentiated CHS. We thus evaluated the impact of anti-inflammatory immune cells (CD68+ TAMs) and pro-inflammatory immune cells (CD8+ TILs) on patient survival. As such, the TAM/TIL ratio was validated as a poor prognostic factor in other cancers. For instance, a high CD68/CD3 ratio in muscle invasive bladder cancer and a higher proportion of CD163+ macrophages than CD8+ lymphocytes in conventional CHS were

associated with poor survival [32,18]. In our study, the dedifferentiated CHS cohort with a high CD68/CD8+ ratio had a poorer survival rate, indicating that the balance between TAMs and pro-inflammatory TILs is tightly correlated with CHS progression.

Since dedifferentiated CHS is highly metastatic, we evaluated the relationship between immune infiltrate and metastatic spreading. Dedifferentiated CHS presenting a high density of CD68+ TAMs had a shorter metastasis progression free survival (7 months vs 36 months). Besides, 100% of patients displaying high CD68+ and CD163+ TAMs presented metastases at diagnosis. As the presence of CD68+ and CD163+ cells were tightly linked, this suggests that a subgroup of macrophages could be related to the initiation of metastases. Indeed, TAMs are known to be involved in the development of metastases in other tumors through their role in angiogenesis, migration and invasion [33,34]. The high CD68+/CD3+CD20 ratio was correlated with poorer distant relapse-free survival in breast cancer [35] and the presence of CD68+ TAMs was correlated with microvascular density in squamous cell carcinoma [36].

By considering the histological type of the dedifferentiated compartment of dedifferentiated CHS, we highlighted a higher density of CD68+ TAMs and a higher ratio of CD68/CD8 in the osteosarcoma subtypes than in others. Moreover, these patients were the only ones to display an association between a higher density of CD68+ TAM and poor survival. These results suggest that the primordial role of CD68+ TAMs on the progression of dedifferentiated CHS is particularly important in dedifferentiated CHS with an osteosarcoma dedifferentiated compartment. Nevertheless, in osteosarcoma, a high density of CD68+ TAMs is generally associated with better survival [10,12].

Other actors like ICPs are involved in the immunosuppressive balance by regulating the anti-tumoral immune response. We thus evaluated the expression of some of those in our conventional and dedifferentiated CHS cohorts [37]. In our cohorts, transcriptomic analyses showed a higher expression of ICPs present on APC such as *CSF1R*, *B7H3*, *SIRPA*, than those linked to lymphocytes: *PD1/PDL1*, *LAG3*. Macrophages being the major immune population encountered in CHS, these results are not surprising. Interestingly, all these ICPs are known to promote an immunosuppressive environment. These findings substantiate our results, further emphasizing the fact that the immune environment of CHS leans towards an immunosuppressive environment. Hence, we actively sought other immunosuppressive actors, including (i) CSF1R, which promotes macrophage recruitment, survival and proliferation, and is expressed on TAMs, the most representative immune cell in both CHS subtypes, and (ii) PDL1 (/PD1) since its expression was already evaluated in a previous study showing PDL1 positivity for more than 50% of dedifferentiated CHS [17].

Thus we validated the expression of PD1/PDL1 and CSF1R by IHC. As previously reported, we confirmed that PDL1 expression is restricted to dedifferentiated tumor cells (42% of patients being positive) [17], reinforcing a rationale for testing anti-PDL1 antibodies in dedifferentiated CHS. PD1, the ligand of PDL1, was expressed in both subtypes of CHS. The anti-PD1 antibody (pembrolizumab) has already been tested in dedifferentiated CHS in the SARCO28 phase II study and caused partial response in 1 out of 5 dedifferentiated CHS patients enrolled [38]. The anti-PD1 therapy could be further tested in dedifferentiated but not in the conventional subtypes since the ligand is absent. We evaluated PDL1 expression on tumor cells but PDL1 could also be expressed on immune cells such as TAMs. Recent studies highlighted the importance of PDL1+ TAMs in tumor progression and response to immunotherapies in many cancers [39–41]. To fully characterize PDL1 in CHS, A co-staining of CD68 and PDL1 could enable us to better appreciate the role of this ICP in CHS.

The most innovative finding in our study, is the high expression of CSF1R, a common potential target in 2 CHS subtypes. Of note, a clinical trial testing CSF1R inhibitors in soft tissue sarcoma is ongoing [15]. Even if CSF1R was expressed in both CHS subtypes, a higher density of CSF1R+ TAMs was found in dedifferentiated subtypes compared with

conventional subtypes. However no correlation between the expression of CSF1R and overall survival was found [37], unlike hepatocellular carcinoma and clear cell renal carcinoma, in which a high expression of CSF1R was associated with poor outcome [42,43]. A larger cohort of patients could be helpful to better understand the role of TAMs CSF1R + in CHS progression.

Interestingly, in our study, a high density of CSF1R+ TAMs was found in dedifferentiated metastatic CHS at diagnosis, suggesting that the presence of those macrophages could participate to metastasis development as it was highlighted in breast cancer [34].

The results obtained in this study should be confirmed using a larger cohort regrouping cases of different centers in Europe.

Our results argue in favor of targeting TAMs in the immune environment of CHS to improve patient survival and decrease the development of metastases. Macrophage-targeting therapies are being developed: they aim at blocking the recruitment of monocytes (anti-CCL2,-CCR2,-CD11b), decreasing the activation of TAMs (CSF1R inhibitor, Trabectedin®), and reprogramming TAMs into pro-inflammatory macrophages (mifamurtide) [44]. Two options could be envisioned in CHS treatment: CSF1R inhibitors could block the activation of the high number of CSF1R+ TAMs, while mifamurtide could induce TAM reprogramming into a pro-inflammatory phenotype rather than an immunosuppressive one. Those different options could be tested in an immunocompetent model, such as the Swarm Rat Chondrosarcoma model, mimicking a grade 2 conventional CHS.

In conclusion, our results converge to indicate that CHS is able to create an immunosuppressive environment in favor of its progression. Anti-tumor immunity may be regained by turning the immunosuppressive environment into a pro-inflammatory environment using immunomodulation through macrophages such as CSF1R inhibitors and/or ICP inhibitors such as anti-PDL1 in the dedifferentiated subtype.

Funding

This work was supported by Cancerpole Auvergne Rhone Alpes (Oncostarter Grant) and the French national academy of pharmacy (Élie Bzoura award).

Declaration of Competing Interest

The authors declare that there are no conflicts of interest

Acknowledgments

B. Manship provides writing assistance.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jbo.2019.100271.

References

- H.L. Evans, A.G. Ayala, M.M. Romsdahl, Prognostic factors in chondrosarcoma of bone. A clinicopathologic analysis with emphasis on histologic grading, *Cancer* 40 (2) (1977) 818–831.
- V.Y. Jo, C.D.M. Fletcher, WHO classification of soft tissue tumours: an update based on the 2013 (4th) edition, *Pathology* 46 (2) (2014) 95–104.
- F.Y. Lee, et al., Chondrosarcoma of bone: an assessment of outcome, *J. Bone Jt. Surg. Am.* 81 (3) (1999) 326–338.
- J. Björnsson, R.A. McLeod, K.K. Unni, D.M. Ilstrup, D.J. Pritchard, Primary chondrosarcoma of long bones and limb girdles, *Cancer* 83 (10) (1998) 2105–2119.
- A.Y. Giuffrida, J.E. Burgueno, L.G. Koniaris, J.C. Gutierrez, R. Duncan, S.P. Scully, Chondrosarcoma in the United States (1973 to 2003): an analysis of 2890 cases from the SEER database, *J. Bone Jt. Surg. Am.* 91 (5) (2009) 1063–1072.
- H. Gelderblom, et al., The clinical approach towards chondrosarcoma, *The Oncologist* 13 (3) (2008) 320–329.
- G. Polychronidou, V. Karavasilis, S.M. Pollack, P.H. Huang, A. Lee, R.L. Jones, Novel therapeutic approaches in chondrosarcoma, *Fut. Oncol.* 13 (7) (2017) 637–648.
- M.J.M. Gooden, G.H. de Bock, N. Leffers, T. Daemen, H.W. Nijman, The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis, *Br. J. Cancer* 105 (1) (2011) 93–103.
- B. Fritzsche, et al., CD8(+)/FOXP3(+)-ratio in osteosarcoma microenvironment separates survivors from non-survivors: a multicenter validated retrospective study, *Oncoimmunology* 4 (3) (2015) e990800.
- P. Koirala, et al., Immune infiltration and PD-L1 expression in the tumor micro-environment are prognostic in osteosarcoma, *Sci. Rep.* 6 (2016) srep30093.
- E.P. Buddingh, et al., Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating agents, *Clin. Cancer Res.* 17 (8) (2011) 2110–2119.
- A. Gomez-Brouchet, et al., CD163-positive tumor-associated macrophages and CD8-positive cytotoxic lymphocytes are powerful diagnostic markers for the therapeutic stratification of osteosarcoma patients: An immunohistochemical analysis of the biopsies from the French OS2006 phase 3 trial, *Oncoimmunology* 6 (9) (2017).
- J.R. Castellanos, et al., B7-H3 role in the immune landscape of cancer, *Am. J. Clin. Exp. Immunol.* 6 (4) (2017) 66–75.
- C.E. Lewis, J.W. Pollard, Distinct role of macrophages in different tumor micro-environments, *Cancer Res.* 66 (2) (2006) 605–612.
- M.A. Cannarile, M. Weisser, W. Jacob, A.-M. Jegg, C.H. Ries, D. Rüttinger, Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy, *J. Immun. Therapy Cancer* 5 (2017) 53.
- M.A. Morrissey, R.D. Vale, CD47 Suppresses Phagocytosis by Repositioning SIRPA and Preventing Integrin Activation, *bioRxiv*, 2019752311.
- M. Kostine, A.H. Cleven, N.F.C.C. de Miranda, A. Italiano, A.-M. Cleton-Jansen, J.V.M.G. Bovée, Analysis of PD-L1, T-cell infiltrate and HLA expression in chondrosarcoma indicates potential for response to immunotherapy specifically in the dedifferentiated subtype, *Mod. Pathol.* 29 (9) (2016) 1028–1037.
- F.A. Simard, et al., Description of the immune microenvironment of chondrosarcoma and contribution to progression, *Oncoimmunology* 6 (2) (2017) e1265716.
- J. Barretina, et al., The Cancer Cell Line Encyclopedia enables predictive modeling of anticancer drug sensitivity, *Nature* 483 (7391) (2012) 603–607.
- N. Leffers, et al., Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer, *Cancer Immunol. Immunother.* 58 (3) (2009) 449–459.
- L. Yang, Y. Zhang, Tumor-associated macrophages: from basic research to clinical application, *J. Hematol. Oncol.* 10 (1) (2017) 58.
- T. Ding, et al., High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection, *Hum. Pathol.* 40 (3) (2009) 381–389.
- C.-X. Ren, et al., Intratumoral and peritumoral expression of CD68 and CD206 in hepatocellular carcinoma and their prognostic value, *Oncol. Rep.* 38 (2) (2017) 886–898.
- C.-Y. Su, X.-L. Fu, W. Duan, P.-W. Yu, Y.-L. Zhao, High density of CD68+ tumor-associated macrophages predicts a poor prognosis in gastric cancer mediated by IL-6 expression, *Oncol. Lett.* 15 (5) (2018) 6217–6224.
- K. Sugimura, et al., High infiltration of tumor-associated macrophages is associated with a poor response to chemotherapy and poor prognosis of patients undergoing neoadjuvant chemotherapy for esophageal cancer, *J. Surg. Oncol.* 111 (6) (2015) 752–759.
- Y. Morita, et al., Pathologic evaluation of tumor-associated macrophage density and vessel inflammation in invasive breast carcinomas, *Oncol. Lett.* 14 (2) (2017) 2111–2118.
- L.A. Elliott, G.A. Doherty, K. Sheahan, E.J. Ryan, Human tumor-infiltrating myeloid cells: phenotypic and functional diversity, *Front. Immunol.* 8 (2017).
- M. Petrillo, et al., Polarisation of tumor-associated macrophages toward M2 phenotype correlates with poor response to chemoradiation and reduced survival in patients with locally advanced cervical cancer, *PLoS One* 10 (9) (2015) e0136654.
- Y. Yao, X.-H. Xu, L. Jin, Macrophage polarization in physiological and pathological pregnancy, *Front. Immunol.* 10 (2019).
- M. Kostine, et al., Increased infiltration of M2-macrophages, T-cells and PD-L1 expression in high grade leiomyosarcomas supports immunotherapeutic strategies, *Oncoimmunology* 7 (2) (2017).
- E.P. Buddingh, et al., Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating agents, *Clin. Cancer Res.* 17 (8) (2011) 2110–2119.
- G. Sjö Dahl, et al., Infiltration of CD3+ and CD68+ cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors. Grant support: The Swedish Cancer Society, the Swedish research council, the Nilsson Cancer foundation, the BioCARE Strategic Cancer Research program, the Lund Medical Faculty, and FoU Landsting Kronoberg and Södra Regionvårdsnämnden. *Urol. Oncol. Semin. Orig. Investig.* 32 (6) (2014) 791–797.
- S. Aras, M.R. Zaidi, TAMEless traitors: macrophages in cancer progression and metastasis, *Br. J. Cancer* 117 (11) (2017) 1583–1591.
- N. Linde, et al., Macrophages orchestrate breast cancer early dissemination and metastasis, *Nat. Commun.* 9 (1) (2018) 21.
- N. Eiró, et al., Impact of CD68/(CD3+CD20) ratio at the invasive front of primary tumors on distant metastasis development in breast cancer, *PLoS One* 7 (12) (2012).
- S. Sun, X. Pan, L. Zhao, J. Zhou, H. Wang, Y. Sun, The expression and relationship of CD68-tumor-associated macrophages and microvascular density with the prognosis of patients with laryngeal squamous cell carcinoma, *Clin. Exp. Otorhinolaryngol.* 9 (3) (2016) 270–277.
- C. Donini, L. D'Ambrosio, G. Grignani, M. Aglietta, D. Sangiolo, Next generation

- immune-checkpoints for cancer therapy, *J. Thorac. Dis.* 10 (Suppl 13) (2018) S1581–S1601.
- [38] H.A.-H. Tawbi, et al., Safety and efficacy of PD-1 blockade using pembrolizumab in patients with advanced soft tissue (STS) and bone sarcomas (BS): results of SARC028—a multicenter phase II study. *JCO* 34 (15_suppl) (2016) 11006–11006.
- [39] M. Pollari, et al., PD-L1 + tumor-associated macrophages and PD-1 + tumor-infiltrating lymphocytes predict survival in primary testicular lymphoma, *Haematologica* 103 (11) (2018) 1908–1914.
- [40] G.P. Hartley, L. Chow, D.T. Ammons, W.H. Wheat, S.W. Dow, Programmed cell death ligand 1 (PD-L1) signaling regulates macrophage proliferation and activation, *Cancer Immunol. Res.* 6 (10) (2018) 1260–1273.
- [41] H. Xiong, et al., Anti-PD-L1 treatment results in functional remodeling of the macrophage compartment, *Cancer Res.* 79 (7) (2019) 1493–1506.
- [42] J.-B. Jia, et al., High expression of macrophage colony-stimulating factor-1 receptor in peritumoral liver tissue is associated with poor outcome in hepatocellular carcinoma after curative resection, *Oncologist* 15 (7) (2010) 732–743.
- [43] L. Yang, et al., Increased expression of colony stimulating factor-1 is a predictor of poor prognosis in patients with clear-cell renal cell carcinoma, *BMC Cancer* 15 (2015) 67.
- [44] L. Yang, Y. Zhang, Tumor-associated macrophages, potential targets for cancer treatment, *Biomark Res.* 5 (2017).