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François Simard

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François Simard

Implication of immune system in chondrosarcoma progression and therapeutic response. Could immunotherapy play a role in chondrosarcoma treatment ?

Devant le jury composé de:

Président	M.DUMONTET, Charles	PU-PH, Université Lyon I, HCL
Rapporteurs	Mme.BOVEE, Judith M.BLANCHARD, Frederic	Md/PhD, Leiden University Medical Center PhD/DR2, INSERM, Université de Nantes
Examineurs	M.DUMONTET, Charles M. GROSSIN Laurent	PU-PH, Université Lyon I, HCL PhD, CR1, Université Lorraine
Co-directrice	Mme.DUTOUR, Aurélie	PhD, Cadre biologiste, CLB Lyon
Directeur	M. BLAY, Jean-Yves	MD/Phd, Directeur Centre Léon Bérard

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Ce travail a été réalisé au sein de l'équipe 11
« Ciblage de la tumeur & environnement immunitaire »

Christophe Caux/ Jean-Yves Blay
CRCL (Cancer Research Center of Lyon)
INSERM UMR 1052, CNRS UMR 5286

Dans les locaux du :

Centre Léon Berard, Batiment Cheney A 1^{er} étage, 28 rue Laennec,
Lyon, France



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Résumé

Introduction

Les chondrosarcomes représentent 20% de tous les sarcomes osseux. Ces tumeurs dont l'incidence est estimée à 1/200 000 par an, sont caractérisées par la production d'une matrice cartilagineuse par les cellules tumorales, une faible vascularisation et un faible pourcentage de cellules en division. Du fait notamment de l'abondance de matrice extracellulaire et de leur faible densité vasculaire, les chondrosarcomes sont particulièrement chimio- et radio-résistants. En conséquence, la prise en charge thérapeutique de ces tumeurs n'a que peu évolué au cours des 30 dernières années et le seul traitement efficace à l'heure actuelle reste une résection chirurgicale large de la tumeur. Des approches d'immunothérapies ont récemment généré un grand enthousiasme dans la recherche anticancéreuse ; ces thérapies exploitent la capacité de l'immunité innée et adaptative pour contenir la croissance de cellules cancéreuses et les détruire. Les points de contrôle « checkpoints » immunitaires sont des protéines de signalisation permettant le maintien de la tolérance du soi et de limiter les réponses immunitaires physiologiques dans les tissus périphériques ; détournés de leur fonction, ils peuvent également permettre l'immunotolérance vis-à-vis des tumeurs. Des inhibiteurs de ces checkpoints immunitaires (Cytotoxic T lymphocyte antigen 4 (CTLA-4) ou de programmed cell death protein 1 (PD-1) et son ligand (PD-L1) induisent des réponses immunitaires contre les cellules cancéreuses, in vivo chez l'homme. Ils restaurent l'activité antitumorale des cellules immunocompétentes et pourraient augmenter l'immunogénicité tumorale induite par les thérapies conventionnelles (chimiothérapie, radiothérapie) ou les thérapies ciblées. L'impact de ces immunothérapies est mal connu dans les sarcomes, et notamment le chondrosarcome. Il apparaît donc intéressant d'étudier l'environnement immunitaire du chondrosarcome et d'évaluer les possibilités de combiner des

inhibiteurs de checkpoints immunitaires à des thérapies de première ligne, comme les inhibiteurs de la voie PI3K/Akt/mTOR.

Jusqu'à présent, très peu d'études se sont intéressées à l'environnement immunitaire du chondrosarcome et à son implication dans la progression et la réponse tumorales. Notre groupe a construit un projet de recherche s'intéressant à ces questions et explorant le rôle joué par le checkpoint immunitaire PD1/PDL1 dans la progression de cette tumeur afin de déterminer si PD1 et PDL1 peuvent être de nouvelles cibles thérapeutiques pour les patients atteints de chondrosarcome et s'ils peuvent être joint à des thérapies de première ligne. Ci-dessous, sont présentés les résultats obtenus jusqu'à présent.

Résultats

1- Caractérisation du microenvironnement immunitaire du chondrosarcome

Nous avons analysé le microenvironnement immunitaire du chondrosarcome à la fois dans des échantillons de chondrosarcome humains (26 échantillons) et dans le modèle de rat de chondrosarcome Swarm (SRC).

Les analyses immunohistochimiques réalisées à l'aide de marqueurs des lymphocytes (CD3, CD4, CD8) et des macrophages (CD163) ont permis de mettre en évidence la présence d'infiltrats immunitaires (lymphocytes et macrophages) en périphérie des nodules cartilagineux dans la zone fibreuse de la tumeur. Nous avons pu montrer qu'une forte présence des lymphocytes T (CD3+, CD4+, CD8+) est corrélée à une meilleure survie et un moindre taux de rechutes des patients. A l'inverse, une corrélation inverse entre la présence de macrophages (CD163+) et le taux de survie des patients est observée. Le ratio cellules CD8+/cellules CD163+ est inversement corrélé à l'agressivité de la tumeur : dans les tumeurs de faible grade et non invasives une plus forte proportion de cellules CD8+ par rapport aux cellules CD163+ est retrouvée alors

que les chondrosarcomes invasifs de haut grade présentent une faible proportion de cellules T CD8+ et une plus forte proportion de cellules CD163+. Ceci indique un rôle potentiel pour les macrophages et les lymphocytes T CD8+ dans l'invasion du chondrosarcome ; les cellules CD163+ favoriseraient la progression tumorale alors que les cellules T CD8+ la contiendraient.

Des analyses similaires ont été réalisées dans le modèle de chondrosarcome de Swarm. Ces études ont montré un profil d'infiltration immunitaire de cette tumeur similaire à celui observé pour le chondrosarcome humain, avec une présence de lymphocytes (CD3, CD8) et de macrophages (CD163) à la périphérie de la tumeur et dans les interstices fibreux. Comme chez l'humain, le ratio CD8+/CD163+ a été calculé, il est inversement corrélé à la progression tumorale et confirme le rôle potentiel des macrophages CD163+ et des lymphocytes T CD8+ dans la progression de chondrosarcome. Par ailleurs, nous avons confirmé le rôle de cellules immunitaires dans le contrôle de la progression tumorale. Des études de cytotoxicité réalisées à l'aide de splénocytes de rats sains ou porteurs de tumeurs ont montré que la présence de la tumeur induit une réponse immunitaire anti cancer chez le rat. En effet, une action cytotoxique spécifique est observée pour les splénocytes de rats porteurs de chondrosarcome (cytotoxicité de 27% contre des chondrocytes à un ratio effecteur cible de 100/1), réponse qui n'est pas obtenue avec des splénocytes de rats sains.

2- Rôle des différentes populations immunitaires dans la progression du chondrosarcome

La présence de macrophages semble contribuer à la croissance du chondrosarcome, tandis que celle des lymphocytes semble associée à un ralentissement de la croissance tumorale. Afin de vérifier cette hypothèse, nous avons inhibé l'une après l'autre ces populations immunitaires dans le modèle de chondrosarcome de rat. Ces traitements ont été administrés pendant 5 semaines

sur des rats porteurs de tumeur progressive, et montrent qu'en absence de lymphocytes T les tumeurs progressent significativement plus vite. A l'inverse, en l'absence de macrophages M2, un ralentissement significatif de la progression tumorale est observé.

Ces données indiquent que les macrophages (principalement les macrophages M2) exercent une action protumorale et pourraient être une cible d'intérêt pour les futures approches thérapeutiques. Ces résultats confirment également le rôle des lymphocytes T dans le contrôle de la croissance du chondrosarcome et cette action antitumorale pourrait être améliorée par des approches d'immunothérapie ciblant les checkpoints immunitaires.

3- Effet des inhibiteurs de la voie PI3K/Akt/mTOR sur la réponse immunitaire

Les inhibiteurs de la voie PI3K/Akt/mTOR réduisent significativement la progression tumorale dans le modèle de chondrosarcome de rat. De plus, ils n'affectent pas l'infiltration des populations immunes (macrophages et lymphocytes) dans le microenvironnement de la tumeur et une diminution de l'expression de PD-L1 dans les tumeurs de rats et dans des lignées cellulaires de sarcomes humains.

Par contre, les inhibiteurs testés réduisent la cytotoxicité des splénocytes de rat face à des cellules de chondrosarcomes de rat. Ils ont aussi comme effet de réduire la sécrétion de cytokine pro-inflammatoire au niveau systémique.

Cela suggèrent que les inhibiteurs de la voie PI3K/Akt/mTOR ont un effet immunosuppresseur et qu'ils seraient inopportun des les associer à une immunothérapie.

4- Checkpoint immunitaire PD-1/PD-L1 et le chondrosarcome

Nous avons étudié l'expression de PD-1/PD-L1 dans le chondrosarcome humain et de rats. Ce checkpoint immunitaire joue un rôle dans l'échappement tumoral. En effet, au niveau tumoral, la liaison de PD-1 et PD-L1 inhibe la prolifération de lymphocytes T cytotoxique (CTL), leur production de cytokines et leur cytotoxicité, favorisant ainsi la prolifération tumorale.

Nous avons mis en évidence l'expression de PDL1 par les cellules tumorales à la fois dans les échantillons de chondrosarcome humain et dans le modèle de chondrosarcome de Swarm. Des cellules immunitaires PD1+ ont été trouvées à la fois dans le chondrosarcome humain et de rat.

Ces résultats montrent qu'une immunothérapie visant les « immuno-checkpoints » PD-1/PD-L1 est possible pour le chondrosarcome. Nous avons aussi démontré que l'étude des populations qui infiltrent le chondrosarcome pourrait servir de biomarqueur potentiel pour ces patients. Nous étudions actuellement le rôle de PD-1/PD-L1 dans le chondrosarcome à l'aide du modèle de chondrosarcome de Swarm en utilisant un anticorps bloquant PD-L1. Nous allons également confirmer les observations conduites dans les chondrosarcomes humains en augmentant la cohorte de patients analysés et étendre l'étude aux sous types plus rares de chondrosarcomes.

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List of abbreviations

ABCB1	ATP binding cassette subfamily B member 1
ACT	Adoptive cell transfer
APC	Antigen presenting cell
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma-extra large
CD	Cluster of differentiation
CHS	Chondrosarcoma
CTA	Cancer testis antigen
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbant assay
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FDA	Food and drug association
HDAC	Histone deacetylase
HH	Hedgehog
HIF-1 α	Hypoxia induced factor-1 alpha
HLA	human leukocyte antigen
IDH	Is citrate dehydrogenase
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
KIR	Killer Ig-like receptor
L-MTP-PE	liposomal muramyl tripeptide phosphatidylethanolamine
M1	Macrophage type 1
M2	Macrophages type 2
MCA	Methylcholanthrene
MHC	Major histocompatibility complex
mTOR	mammalian target of rapamycin
NK	Natural killer
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PFS	Progression free survival
PI3K	Phospholinositide 3-kinase
RT	Radiation therapy
SEER	Surveillance, Epidemiology, and End Results
SRC	Swarm rat chondrosarcoma
TAA	Tumor associated antigen
TAM	Tumor associated macrophage
TCR	T cell receptor
TGF	Transforming growth factor
Th	Lymphocyte T helper type 1
Treg	Lymphocyte T regulator
ZOL	Zoledronic acid

1. Chondrosarcoma: A rare tumor and a therapeutic challenge

Chondrosarcoma encompasses a group of tumors characterized by chondroid matrix production that consist predominantly of cartilage and that range from low-grade tumors with low metastatic potential to high-grade, aggressive tumors characterized by early metastasis. Chondrosarcoma is defined as a “locally” aggressive or malignant group of cartilaginous matrix producing neoplasm with diverse morphological features and clinical behavior characteristics (WHO Classification) (1).

In the following paragraphs we will present the different types of chondrosarcoma, their conventional treatments as well as the therapeutic options being currently explored. Given the heterogeneity of this group of tumor we will focus on the most encountered chondrosarcoma: the conventional central chondrosarcoma.

1.1 Epidemiology

The estimated overall incidence of CHSs is 1 in 200,000 per year (2), and it is the third most frequent malignant bone tumor after multiple myeloma and osteosarcoma. It is estimated that CHSs account for approximately 20~30% of primary malignant bone tumors (3).

Chondrosarcoma most commonly arise in adults aged 30 to 70 years, with a peak around 40 to 60. Chondrosarcoma has a male to female ratio of 1.5 to 1. It is most common in the femur, humerus, ribs and on the surface of the pelvis.

1.2 Risks factors

Most often, chondrosarcoma happens from normal cartilage cells. It may also arise from a pre-existing benign bone or cartilage tumor. Patients with enchondroma, with Ollier's disease (multiple enchondromatosis) or Maffucci's syndrome (multiple enchondromas and hemangiomas) are at much higher risk of chondrosarcoma than the normal population and often present in the third and fourth decade (4).

1.3 Classification

Except for their origin in preexisting benign cartilaginous diseases conditions, secondary chondrosarcomas are similar to conventional chondrosarcomas in many aspects.

Chondrosarcoma is a heterogeneous group of tumor; as a consequence many chondrosarcoma subtypes are described. More than 90% of chondrosarcoma are conventional chondrosarcoma. Among these, 90% are low grade to intermediate grade tumors; the remaining 5-10% are grade 3 lesions with high metastatic potential. Conventional chondrosarcoma are sub-classified as central when they arise within the medullary cavity or as peripheral when they arise from the surface of the bone (Figure 1.1; Figure 1.2)

Besides conventional chondrosarcoma, variants are encountered. They include dedifferentiated chondrosarcoma, mesenchymal chondrosarcoma, clear cell chondrosarcoma, and myxoid chondrosarcoma.

In addition to the morphologic classification, chondrosarcomas can be classified into 3 histologic grades, depending on findings of cellularity, atypia, and pleomorphism:

Grade I (low grade) – Cytologically similar to enchondroma (3);

Grade II (intermediate grade) – Characterized by a definite and increased cellularity (3);

Grade III (high grade) – Characterized by high cellularity, prominent nuclear atypia, and the presence of mitosis (3)

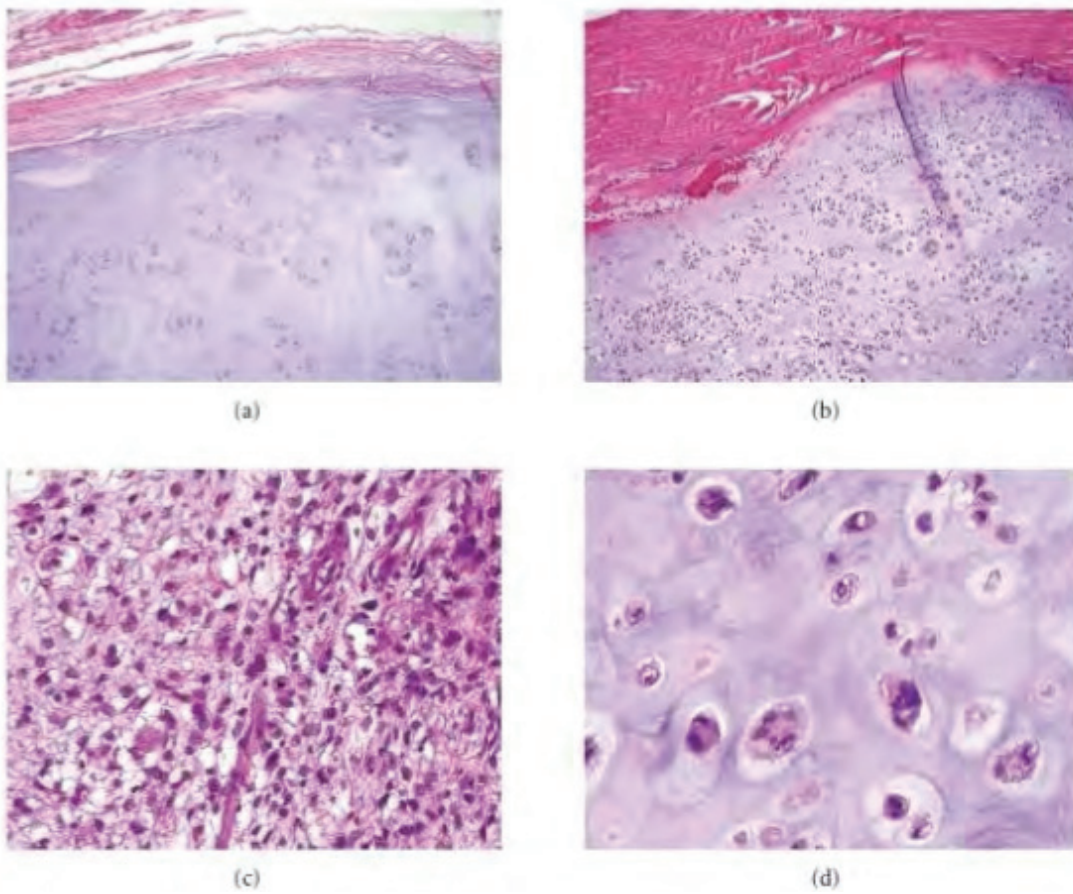


Figure 1.1: Histological aspect of conventional chondrosarcoma. a: Grade 1 chondrosarcoma; b: Grade 2 chondrosarcoma; c and d: Grade 3 chondrosarcoma (2)

The higher the grade, the more likely the tumor is to spread and metastasize. Grade 1 lesions rarely metastasize; whereas 10-15% of grade 2 lesions and more than 50% grade 3 lesions metastasize.

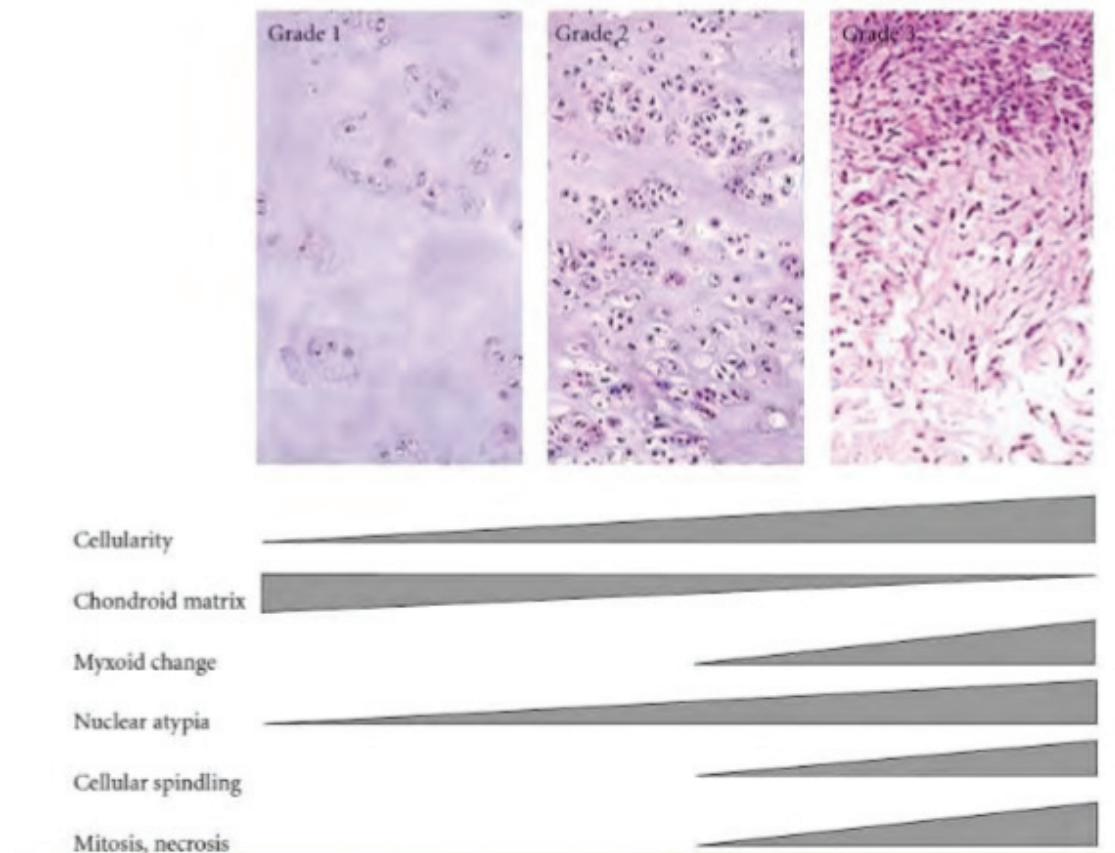


Figure 1.2: Histological grading of chondrosarcomas (2).

1.4 Survival and Prognosis factors

Survival of chondrosarcoma varies considerably across the large number of subtypes and histologic grades of these tumors. Patients with adequately resected low-grade chondrosarcomas have an excellent survival rate. In general the survival of patients with high-grade tumors varies depending on the location, subtype, effectiveness of surgical treatment, size and stage of the tumor (4).

Chondrosarcoma are often slow evolving tumors. Thus the reported 5 years survival rates are higher than most of tumors depend on the grade of chondrosarcoma. The reported 5-year survival for grade 1 chondrosarcoma ranged from 82% to 99%. The 5-year survival for grade 2 chondrosarcoma

ranged from 63% to 92%. The 5-year survival for grade 3 chondrosarcoma ranged from 0% to 77%. The lowest (0%) survival was displayed in a study looking at a very small subgroup of patients treated with an intralesional resection (5).

As no therapeutic treatment besides surgery is available for chondrosarcoma, The 10 years survival rate of this tumor has not evolved in more than 20 years as indicated by the 2 studies conducted in 1977 by Evans and in 2001 by Anract et al (Table 1.1).

10 years survival	Evans 1977(6)	Anract, de Pinieux and al. 2001(7)
Grade I chondrosarcoma	83%	83%
Grade II chondrosarcoma	64%	60%
Grade III chondrosarcoma	29%	5%

Table 1.1: 10 years survival of chondrosarcoma patients in function of the histological grade

Identification of prognostic factors is important to select the optimal treatment strategy. To direct efforts in the development of new therapeutic strategies the identification of proven prognostic factors of central chondrosarcoma is important, even though the treatment options are still currently limited.

Various studies have suggested that histologic grade, size, stage, and anatomic location of the lesion are important prognostic features (8, 9). In an analysis of 2,890 patients with chondrosarcoma from the Surveillance, Epidemiology, and End Results (SEER) database, only grade and stage were identified as independent prognostic factors for survival (10). For Fiorenza and al., on 153

non-metastatic chondrosarcoma patients based on a multivariate analysis only extracompartmental spread and high histologic grade were a risk factor (11).

1.5 Current treatment

The clinical management of chondrosarcoma remains a challenging problem as this is highly resistant to currently available chemotherapy and radiation treatment regimens (2, 12, 13). Surgery remains the cornerstone in CHS treatment. Several studies have demonstrated the benefit of resection of tumor with wide surgical margins (14-16).

1.5.1 Surgery

The goal of chondrosarcoma resection is complete tumor removal with microscopically negative margins. Intralesional surgery (microscopically positive margins) should be avoided because it is associated with a high risk of local recurrence regardless of whether the patient receives perioperative radiation therapy or chemotherapy; if intralesional surgery has been performed, re-excision of the tumor bed is recommended (17). For low-grade chondrosarcomas of the extremities, intralesional surgery is acceptable, as it has been reported to have a low rate of recurrence (18). However, there are a significant number of chondrosarcomas, especially those arising in the pelvis or the spine, that are difficult to remove with a wide surgical margin (19, 20). In these instances, local recurrence is almost inevitable with major function and life threatening consequences.

1.5.2 Conventional therapies applied to chondrosarcoma

A) Radiation therapy

Conventional radiation therapy (RT) has limited to arguably no effect on conventional chondrosarcoma (13, 21). Currently, there is not enough data to

support its routine use in patients with this disease. However, RT may be considered if there is incomplete resection, for palliation of symptoms, in patients with advanced or unresectable tumors, or for mesenchymal chondrosarcoma (4, 13). Proton beam therapy, carbon ion therapy and neutron therapy have however been reported to induce tumor control in inoperable patients (22) (23).

B) Chemotherapy

Different studies have reported the lack of efficacy or limited efficacy of conventional chemotherapy in patients with advanced chondrosarcoma(24). The chondrosarcoma subtypes for which the highest benefits of chemotherapy can be observed are mesenchymal and dedifferentiated chondrosarcoma (24, 25).

Indeed a recent report from the Italiano show an improvement in dedifferentiated chondrosarcoma and mesenchymal chondrosarcoma patients survival that are treated respectively as if they had osteosarcoma and Ewing sarcoma (25). Similar results were reported by Anderson (24).

1.6 Chondrosarcoma Therapeutic resistance

The biological mechanisms of chondrosarcoma resistance to cytotoxic are multifactorial. The low percentage of dividing cells within a poorly vascular matrix, are thought to be involved in chondrosarcoma therapeutic resistance (4).

Indeed, chemotherapies are most effective on rapidly dividing cells while chondrosarcoma are slow progressing tumors and the low vascularity of the tumor limits access of chemotherapeutic drugs to tumor cells (4, 26). Moreover, chondrosarcomas express high levels of the multidrug resistance-associated protein P-glycoprotein, a product of the ABCB1 gene (27) one of the most effective pump efflux for chemotherapies.

The mechanism of radiation resistance in chondrosarcomas is better understood. Radiation resistance may be related to local tissue hypoxia as has been

described in other tumors, the relative low levels of oxygen preventing the formation and propagation of radiation-induced reactive oxygen species that cause single and double strand DNA damage (28). Expression of HIF-1 α by chondrosarcomas in association with increased levels of Bcl-2 can contribute to both radio resistance and chemoresistance (29). p16^{ink4a}, a tumor suppressor protein, also appears to have a role in radiation resistance of chondrosarcomas (13).

Because no systemic therapy has proven effective, chondrosarcoma are like the gastrointestinal stromal tumors of the 1990s, before the rise of targeted therapies and Gleevec. A variety of strategies are being developed to help overcome the limited effectiveness of current radiotherapy and chemotherapy on chondrosarcomas (30). Some of which will be presented in the following chapter.

1.7 New therapeutic approaches for chondrosarcoma

CHS remains a major challenge in both diagnosis and therapy since conventional chemotherapy has proven to be largely ineffective, with no significant progress made for approximately three decades (31).

Improving chondrosarcoma clinical management is therefore a challenging problem and novel therapeutic approaches are needed.

The increasing understanding of chondrosarcoma biology, mechanisms of chondrosarcoma pathogenesis have prompted the development of new therapeutic approaches over the last decade. Some of these approaches are taking advantages of targeting chondrosarcoma environment. Others are targeting molecular pathways involved in tumorigenesis or that have been described as over-activated or mutated in chondrosarcoma or bone sarcoma.

As numerous strategies are being tested, the following paragraphs will present, on an exhaustive way, some of these new therapeutic approaches for chondrosarcoma.

1.7.1 Targeting chondrosarcoma microenvironment

One of the approaches being investigated is to take advantages of phenotypic microenvironmental features of CHS (that have been considered for many years as limitations), instead of fighting them.

Recent scientific data in the microenvironment and molecular pathways involved in CHS have highlighted the role of matrix metalloproteinases, bone niche, hypoxia-inducible factor (HIF), and mTOR (32-34). Treatments targeting tumoral microenvironment have therefore been suggested as complements to surgical excision (30, 31, 35).

1.7.2 Bone resorption osteoclast activity

Chondrosarcoma induce a local osteolytic reaction with bone remodeling at the site of tumor on gross and histopathological specimens, a phenomenon mediated by the action of osteoclasts, monocyte-derived bone resorbing cells (36).

Teams postulated that osteoclasts in the tumor microenvironment could be exploited to prevent tumor-mediated bone destruction and control tumor growth (37).

A few years ago, Heymann's group published the therapeutic relevance of using zoledronic acid (ZOL; an inhibitor of osteoclasts) in the SRC model. They showed that zoledronic acid, directly inhibits SRC cell cycle progression, induces apoptosis, and reduces peri-skeletal tumor growth in the SRC model (38). Zoledronic acid slows down rat primary chondrosarcoma development, recurrent tumor progression after intralesional curettage and increases overall survival (38). Since then, others have reported the promising effects of

bisphosphonates not only on chondrosarcoma proliferation and invasion *in vitro*, in animal models but also in clinical practice (39-42).

1.7.3 Targeting the chondrogenic matrix of CHS to deliver drugs

One strategy is to exploit the chondrogenic nature of the tumor in order to selectively deliver drugs or radioisotopes to tumoral tissue.

This strategy is being explored by team of JM Chezal and E Miot Noirault. They have developed cartilage-targeting compounds for therapeutic or imaging purposes by linking a quaternary ammonium function to anti-inflammatory drugs, chondroprotective agents, cytotoxic agents and ^{99m}Tc chelate. They have shown the capacity of these compounds to localize to the tumor site after *in vivo* administration (43-45).

Regarding the therapeutic potential of these agents, this team generated a quaternary ammonium derivative of melphalan a conventional DNA alkylating agent (Mel-AQ) in order to enhance both the delivery and the specificity of cytotoxic agents in chondrosarcoma tissue (43). They showed *in vitro* that Mel-AQ had a cytotoxic activity against bone tumor lines (HEMC-SS and Saos-2 cell lines), whereas no effect was observed with melanoma cells or chondrocytes. Moreover, Mel-AQ showed its ability to inhibit tumor cell growth *in vivo* using the syngeneic Swarm rat chondrosarcoma (SRC) model. The antitumor efficacy of this agent and its good tolerance in animals make it a promising candidate for chondrosarcoma treatment (46).

1.8 Targeted therapies for chondrosarcoma

Recent advances in the understanding of molecular pathways involved in chondrosarcoma development have suggested several molecular targets for novel adjuvant therapies for the tumor, such as the inhibition of parathyroid hormone-related protein signaling, matrix metalloproteinases (47), p38 mitogen-

activated protein kinase pathways (48), the treatment with peroxisome proliferator-activated receptor- γ ligands (49), hypoxia-inducible factor (HIF), and mTOR (32, 34).

1.8.1 HDAC inhibitors

The transcriptional regulating effects of histone acetylation or deacetylation play important roles in specific gene expressions of chondrocytes (50-52). Moreover it has also be shown that treatment with the histone deacetylase (HDAC) inhibitor trichostatin A enhances the expression of the α 1 chain of type II collagen (COL2A1) and aggrecan in human primary chondrocytes (52).

HDAC inhibitors represent a novel class of antineoplastic agents and several HDAC inhibitors are currently under clinical trial (53). The antitumor activity of a HDAC inhibitor, depsipeptide (FK228, FR901228), for chondrosarcomas has been tested both *in vitro* and *in vivo* given possible implication in cellular differentiation of chondrosarcomas. Depsipeptide induced, cell cycle arrest, and apoptosis in chondrosarcoma cells *in vitro* (54). In addition, HDAC inhibitor modulates the expressions of extracellular matrix genes in chondrosarcoma cells (55). *In vivo*, the effect of these inhibitors on chondrosarcoma progression was evaluated in a murine xenograft model. In these settings it led to a significant inhibition of tumor growth in a dose-dependent manner without toxicities (55).

Another HDAC inhibitor (SAHA) showed similar effect on chondrosarcoma cells proliferation and induced cell autophagy *in vitro*, inhibited tumor progression in *in vivo* model (56). Thus the novel HDAC inhibitors might be a promising tool to use against chondrosarcomas.

1.8.2 Apoptosis and chondrosarcoma: BH3 mimetics for chondrosarcoma treatment

Bcl-2 mediates an anti-apoptotic function principally by blocking Apaf1-mediated caspase-9 activation. Bcl-2 is expressed at a high level in osteosarcoma and in 63%–71% of high-grade conventional chondrosarcoma. For the rare chondrosarcoma subtypes, the team of J Bovee demonstrated Bcl-2 expression in 26% of dedifferentiated chondrosarcomas, while 86% of clear cell chondrosarcomas and 96% of the mesenchymal chondrosarcomas expressed Bcl-2. They additionally evaluated the expression of the Bcl-2 family member Bcl-xl and demonstrated that in dedifferentiated chondrosarcoma, displaying low expression of Bcl-2, high expression of Bcl-xl was found (57).

Moreover, using a large panel of chondrosarcoma cell lines, they were able to demonstrate that chemoresistance in chondrosarcoma is not due to the large amount of extracellular matrix or due to multidrug resistance pump activity, but is caused by the expression of Bcl-2 family members (58). Therefore, Bcl-2 may be a promising target for therapy for chondrosarcoma.

BH-3 mimetics like compounds developed by Abbot (ABT-737 and ABT-263) are synthetic small-molecule inhibitors of anti-apoptotic proteins B-cell lymphoma-2 (Bcl2) and Bcl-xL, were recently reported to have an anti proliferative effect (decrease cell viability) *in vitro* in 2 chondrosarcoma cell lines and improve animal's survival in an xenograft murine model of chondrosarcoma (59).

1.8.3 Hedgehog pathway and chondrosarcoma

In recent years, the Hedgehog (HH) signaling pathway has emerged as a critical determinant of cancer initiation, progression and metastasis of many types of cancers (60, 61). HH signals have been shown to be active in chondrosarcoma (62). In chondrosarcoma, HH signaling is not responsible for malignant transformation but likely plays a role in cartilage tumorigenesis by maintaining tumor cells in a less differentiated but proliferative state (62).

HH pathway can be targeted at different levels. A study focusing on Gli1 showed that knockdown of Gli1 expression by siRNA downregulated the expression of major HH signal pathway members, including Ihh, PTHrP, PTCH1, and SMO. siGli1 decreased the proliferation rate, induced cell cycle G2/M phase arrest by downregulating cyclinB and Rb phosphorylation, thereby rendering chondrosarcoma cells quiescent and preventing tumor progression. These data indicated that for chondrosarcoma, therapeutic effects might be achieved by Gli1 inhibitors (63).

Another effector of HH pathway that was targeted by a synthetic inhibitor is SMO. IPI926 is small-molecule that directly targets SMO and had shown activity in xenograft models of basal cell carcinoma and medulloblastoma before being tested in chondrosarcoma. It was shown to be a very effective antitumor agent in primary chondrosarcoma xenografts (64).

A Phase 2 multicenter trial evaluating the safety and efficacy of IPI-926 in patients with metastatic or locally advanced (unresectable) chondrosarcoma was initiated (clinical trial NCT01310816) and did not showed promising results or proved disappointing (NCT01310816) even if long term non progressive patients are still under treatment (unpublished data).

1.8.4 Inhibitors of IDH 1/2 Mutant Proteins

Isocitrate dehydrogenase (IDH) mutations were recently identified in approximately 50% of chondrosarcomas and drugs are currently under development to inhibit mutant IDH enzymatic activity (65). Isocitrate dehydrogenase proteins consist of three regulatory enzymes (IDH1, IDH2, and IDH3) that catalyze the NADP⁺-dependent oxidative decarboxylation of isocitrate (ICT) into alpha-ketoglutarate (alpha-KG) in the Krebs cycle

Mutations in the *IDH* family of genes recently have been described in patients with a variety of cancers, among which chondrosarcomas (66, 67). Somatic

mutations also have been identified in up to 70% of primary conventional central chondrosarcomas, and 54% of dedifferentiated chondrosarcomas (65, 68, 69).

Mutated IDH1 or IDH2 are active catalytic enzymes. *in vitro* treatment of IDH1 mutant chondrosarcoma cell lines with an IDH1 inhibitor, induced apoptosis, and inhibited tumorigenic activity (70,71). Clinical trials on IDH inhibitors in chondrosarcoma are underway.

1.8.5 mTOR pathway inhibitors

One of the signaling pathways that has been extensively studied over the last years is the PI3K/Akt/mTOR signaling cascade. Mutations and aberrant activations of this pathway effectors are frequently detected in malignancies including sarcoma and have been linked to cancer development, chemoresistance and radiation resistance mechanisms (47). In cancers, PI3K/mTOR promotes cell survival and proliferation, apoptotic resistance and increasing data also link this pathway to tumor invasion and metastasis (25).

The emergence of pharmacological compounds directed against mTOR represents a major breakthrough for cancer-targeted therapies. First-generation mTOR inhibitors, i.e. rapalogs, have shown anticancer activities in routine clinical setting as well as in clinical trials (72-74). Two sets of recent data suggest that the inhibition of mTOR pathway could be an effective systemic treatment for chondrosarcoma.

The first one was generated by the group of Merimsky who showed in a case report then in a clinical trial that the association of a rapalog with cyclophosphamide is efficient in controlling chondrosarcoma progression (75, 76).

The second one concerns preclinical data generated by our group. Using an orthotopic rat chondrosarcoma model, we have shown that RAD001 inhibits

chondrosarcoma proliferation, significantly reduced tumor growth and delayed tumor recurrence (77).

In vivo this agent was able to significantly slow down chondrosarcoma progression in settings where the conventional agent doxorubicin didn't have a therapeutic effect.

The discrepancy between the *in vivo* activity of rapalogs and lack of direct activity on tumors cells *in vitro* seems to indicate that tumor microenvironment plays a role in chondrosarcoma response to rapalog, as it has been reported for other therapeutic agents (78).

The results obtained in this study, prompted us to explore the role played by tumor microenvironment, more precisely by the immune tumor environment, in chondrosarcoma progression and response to conventional and/or targeted therapy.

In order to better understand immune systems populations involvement in chondrosarcoma, the next chapter will present in a summarized way the role of immune system in cancer.

2. Cancer and the immune system in carcinomas and sarcomas

Cancer is a disease of unrestricted proliferation of cells. The uncontrolled growth of cells that characterize cancer can be caused by many factors, such as mutations in oncogenes and tumor suppressor genes as well as virally encoded proteins; elements causing deregulation of the cell cycle.

The accumulation of mutations in oncogenes or tumor suppressor genes, lead to tumorigenesis which endows the mutation-rich cell with the hallmarks typically present in cancer (Figure 2.1). These include, sustained proliferation, resistance to cell death, angiogenesis, evasion of growth suppressors, replicative immortality, genomic instability, escape from immune destruction, tumor-promoting inflammation, deregulated cellular energetics as well as invasive characteristics (79) (figure 2.1).

The involvement of immune system in the cancer has only been in considered for decades. It was confirmed in human tumors since the advent of therapeutic immunosuppression developed in transplant settings and experience in HIV patients, but confirmed as a potential therapeutic strategy only since the beginning of the 80' with a limited efficacy of recombinant cytokines IFN α and IL-2. The introduction of immune checkpoint inhibitors demonstrated the crucial therapeutic potential of immune system manipulation since 5 years.

There has been remarkable progress in both the understanding of the immune system and of ways to turn it on and off to treat cancer. Given these changes in perspective, cancer immunotherapy was *Science* magazine's 2013 advance of the year (80). As a result of this enthusiasm, many immunotherapeutic approaches have been developed and some are now in clinical trials for all type of cancers including sarcomas.

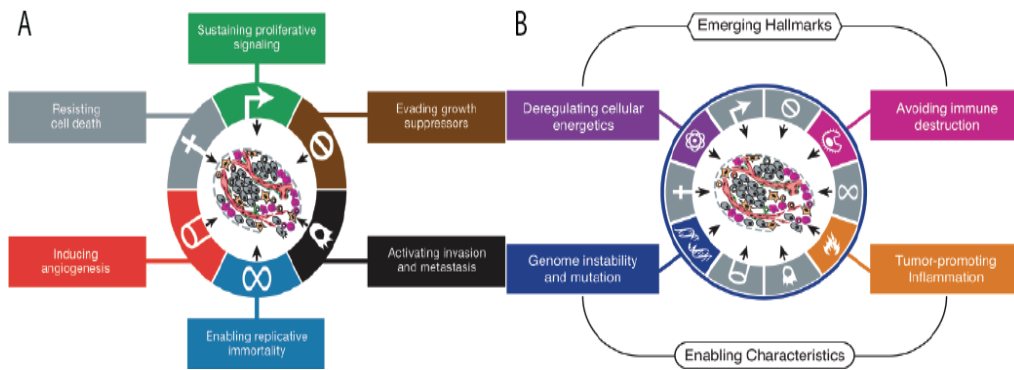


Figure 2.1: Evolution of the hallmarks of cancer over the past decade. A) Cancer hallmarks in 2000. B) Cancer hallmarks in 2011 with novel additions notably of hallmarks of the involvement of the immune system (tumor –promoting inflammation and avoiding immune destruction) (79).

In this section of the introduction, we will briefly present the immune system, its involvement in cancer surveillance. The mechanisms that cancer cell has developed to evade this surveillance will then be introduced and some immunotherapeutic strategies discussed.

Since the knowledge and studies of immune system implication in sarcoma are comparatively way behind what was done in carcinomas, we will only present the immune system in cancer in general with a focus on sarcomas when the data are available and/or different from what is generally accepted in cancer immunity.

Because it is impossible to cover all aspects of the immune response in a single manuscript, we will focus on what is useful for the readers (even non immunologists) to understand the results of this work.

2.1 The immune system in a nutshell

The natural task of the immune system is to protect the human body from infections by bacteria, viruses, and parasites but also against it’s own cells dysfunction.

The immune system is composed of two parallel and closely related components: the innate immunity and the adaptive immunity.

The innate immunity, often called the ‘first line of defense’, defends the host within minutes after exposure to a noxious stimulus. Innate immune response may initiate acute inflammation, which involves the accumulation and activation of phagocytes. The innate immune system uses non-clonal “pattern recognition” receptors (PRRs), which do not recognize human structures but do recognize patterns found on microbes. These receptors recognize pathogen-associated molecular patterns (PAMP), such as single-stranded viral DNA, and as a consequence induce activation and effector functions in innate immune cells. The intruding pathogen will be captured, internalized, and killed by effectors of the innate immunity (macrophages, NK). At the same time, elements of the pathogen, so-called antigens, will be transported from the site of infection to secondary lymphoid organs, such as lymph nodes and the spleen, to be presented to the adaptive arm of the immune system. This presentation is performed by professional antigen-presenting cells (APC), of which dendritic cells (DC) are the most potent (81).

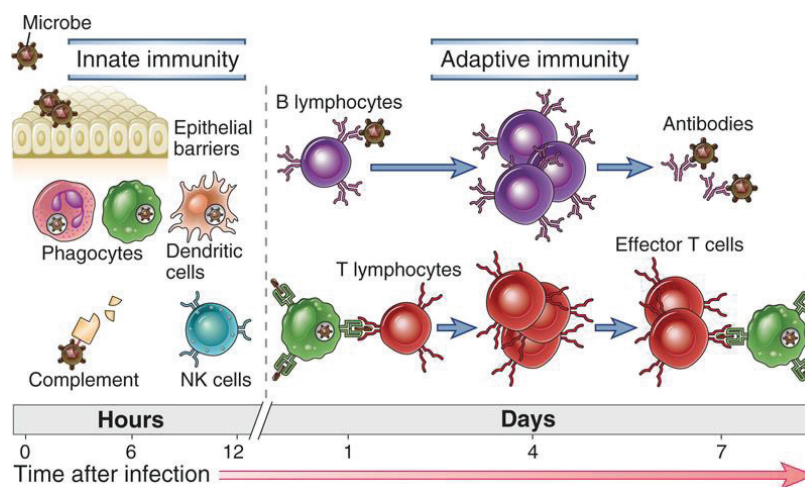


Figure 2.2: Innate and adaptive immunity. The mechanisms of innate immunity provide the initial defense against infections. Adaptive immune responses develop later and consist of activation of lymphocytes. The kinetics of the innate and adaptive immune responses are approximations and may vary in different infections. (81)

The adaptive immunity responses are stimulated by exposure to infectious agents and increase in magnitude with each successive exposure to a particular pathogen. There are two types of adaptive immune responses: humeral immunity mediated by B lymphocytes and cell mediated immunity mediated by T lymphocytes (Figure 2.3).

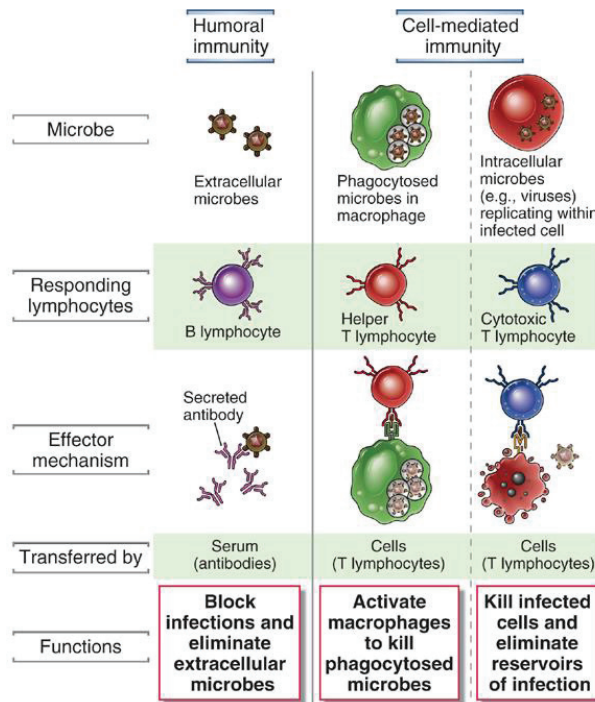


Figure 2.3: The two types of adaptive immune response. Humoral immunity (left) and cell-mediated immunity (right) are resume on this figure. (81)

Each of these cell populations express receptor specific for a particular antigen. A T cell will therefore sample antigens presented by many different DC in the lymph node. The encounter with its cognate antigen will activate this T cell, which will proliferate and produce cytokines. Activated T cells can then migrate to the site of infection in response to chemokines produced by the innate immune cells that first encountered the pathogen (81).

T cells populations are divided into helper T (Th) cells, which are CD4+, and mainly produce cytokines, and 'killer T cells, which express CD8 and can

recognize and kill pathogen-infected cells that present viral antigens on their surface.

Some of the activated, antigen-specific lymphocytes will persist as memory T cells even after the infection is cleared and can be rapidly re-activated if they encountered the same antigen/pathogen.

Immune system function to protect body from aggressions and cellular dysfunction; whether its role was also to contain non virally induced tumors was a matter of debate. The concept of immune surveillance, which was proposed in the 1950s states that a physiological function of the immune system is to recognize and destroy clones of transformed cells. The following paragraphs will present the actors of tumor immune response and mechanisms of immunosurveillance and immunoediting.

2.2 Tumoral immune response

Tumor microenvironment is composed of stromal cells, blood vessels, and innate and adaptive immune cells populations. The lasts are inflammatory infiltrate that cause a local chronic inflammation. Infiltrating leukocytes in the tumor microenvironment may exert a dual role on tumor development and progression. Tumor cells can be directly eliminated by the immune cells, which had developed an antitumoral immune response. Tumor cells can also recruit and instruct immune cells to favor tumor growth and progression. The recruited immune cell consists of many players, including innate and adaptive immune populations. Cells that infiltrate cancer and their role are recapitulated in table below.

Cell type	Markers (human)	Function	References
TAM	CD11b ⁺ CD68 ⁺ CD163 ⁺	Classically activated M1 macrophages are proinflammatory and anti-tumorigenic and secrete TH1 cytokines. Alternatively activated M2 macrophages are anti-inflammatory and pro-tumorigenic and secrete TH2 cytokines. TAMs frequently exhibit an M2 phenotype; their presence in tumors supports angiogenesis and invasion.	(2-6)
DC	CD11c ⁺ CD83 ⁺ CD123 ⁺	DCs are monocytic antigen-presenting cells that are derived from the bone marrow. DCs presenting tumor-specific antigens are being developed as vaccines to induce both innate and adaptive immune responses to regress tumors and prevent relapse.	(5-8)
NK cell	CD56 ⁺ CD16 ⁺	NK cells are cytotoxic lymphocytes that can kill stressed cells in the absence of antigen presentation. NK cells detect and kill tumor cells through 'missing-self' activation (loss of healthy cell markers) or 'stress-induced' activation (gain of stressed cell markers).	(2, 6, 9)
T_H cell	CD3 ⁺ CD4 ⁺	CD4 ⁺ T _H cells can be divided into T _H 1 and T _H 2 lineages. T _H 1 cells secrete proinflammatory cytokines and can be anti-tumorigenic. T _H 2 cells secrete anti-inflammatory cytokines and can be pro-tumorigenic. The ratio of T _H 1 to T _H 2 cells in cancer correlates with tumor stage and grade.	(2, 6, 10)
T_{reg} cell	CD4 ⁺ CD25 ⁺ FOXP3 ⁺ CD45RA ⁺	T _{reg} cells have primarily pro-tumorigenic roles by suppressing immunosurveillance; however, their presence in tumors is positively correlated with overall survival in certain cancer types. These divergent roles may be attributed to context-dependent functions or distinct subpopulations that are challenging to identify at present using conventional markers.	(5, 11-13)
T_C cell	CD3 ⁺ CD8 ⁺	CD8 ⁺ cytotoxic T (T _C) cells are effector cells of the adaptive immune system. They specifically recognize and destroy cancer cells through perforin- and granzyme-mediated apoptosis.	(2, 6, 14)
B cell	CD19 ⁺ CD20 ⁺	B lymphocytes are important mediators of humoral immunity. In cancer, they can promote disease progression by secreting pro-tumorigenic cytokines and altering TH1-to-TH2 ratios. Their importance in supporting tumor growth is evident in B cell-deficient mice, which exhibit resistance to engraftment of certain syngeneic tumors.	(2, 5, 15)

Table 2.1: The Principal immune cells population in cancer

2.2.1 Innate immune response

Cells from the innate immunity can directly recognize cancer cells via specific receptors (resume on figure 2.4) and participate to lymphocytes recruitment to induce an anti-tumoral action (Fig 2.4). Here we have done a short review on the role of the three principal innate immunity effectors within cancer microenvironment: macrophages, natural killer cells and dendritic cells.

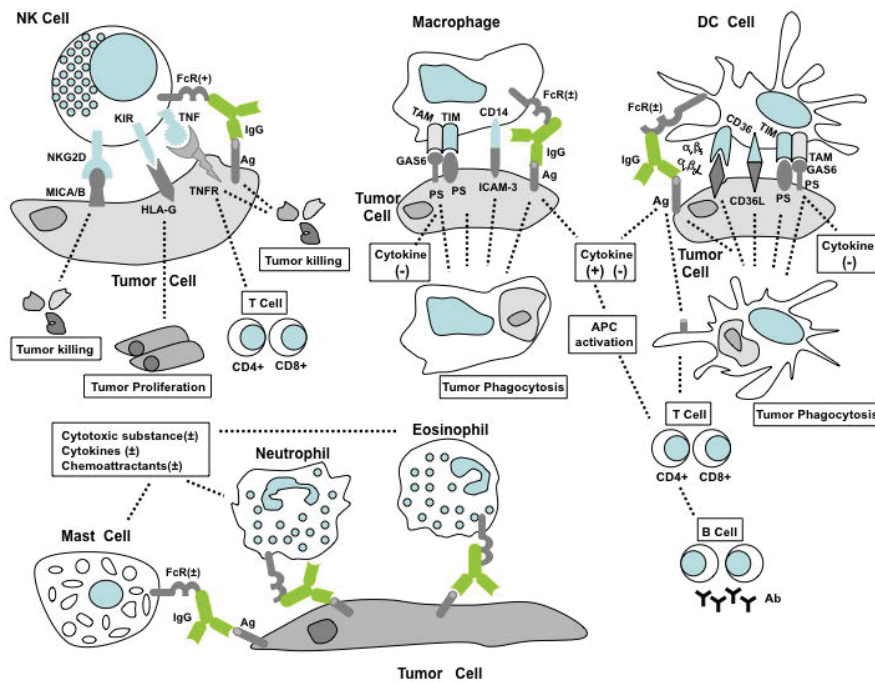


Figure 2.4: Innate immunity and Cancer. NK, macrophages, DC, neutrophils, eosinophils, and mast cells are effectors of the innate immunity: NK cells can cause tumor cell death through the interaction of receptors expressed by NK cells with ligand expressed on tumor cells (like NKG2D and MICAB) or ADCC mechanism. NK cells can also stimulate adaptive immunity or induce tumor cell proliferation. Macrophages phagocytose apoptotic cancer cells via interaction of receptors expressed on macrophages (TAM TIM CD14) and ligand expressed on tumor cells (PS ICAM3). DC phagocyte apoptotic tumor cells. Cytokine secreted by DC promote antigen presenting cell activation, leading to cellular and humoral adaptive immune response (82).

A) Macrophages

Plasticity is a well-known characteristic of macrophages: they are able to adapt their phenotypes in function of signals they receive from the microenvironment (83). Tumor associated Macrophages (TAM) are divided into two antagonistic populations: M1 and M2 macrophages.

M1 macrophages are induced by the exposure to Th1 cytokines (e.g., IFN- γ) alone or in combination to microbial components (e.g., LPS). M1 macrophages participate in Th1 response and are mediators of resistance against tumor cells. Typically M1 polarized macrophages are producers of effector molecules (e.g., reactive oxygen and nitrogen intermediates) and inflammatory cytokines (e.g., IL-1 β , TNF, IL-6). They produce high levels of IL-12, and display a low expression of IL-10(83). In general, cytokines produced by M1 in cancer induce an anti-tumoral response.

The cytokines IL-4 and IL-13 inhibit this classical activation and induce the alternative M2 form of macrophage polarization (84). Unlike M1, M2 macrophages produce high amounts of IL-10 and low amounts of IL-12, express CD14 and CD163 surface proteins and display poor antigen presenting capacity (85). Moreover, they suppress Th1 adaptive immunity, participate in the resolution of inflammation, and promote wound healing, angiogenesis, and tissue remodeling (86). Immunological stimuli, such as IFN- γ or IFN- α , can reprogram immunosuppressive M2 macrophages into immunostimulatory M1 cells (87, 88).

Prognostic significance of TAM in humans has been recently evaluated (89). Though there are evidences that in many cancers, such as breast, cervix, bladder, and gastric carcinoma, high numbers of TAM are significantly associated with poor prognosis, (90). Nevertheless, other studies have reported that the prognostic significance of TAM can be controversial (89). For instance, in high-grade osteosarcoma high infiltration of CD68+ TAM (M1 and M2) are associated with reduced metastasis and improved survival (91), whereas in classical Hodgkin's lymphoma and, large B-cell lymphoma this correlation was not found (92, 93). However a recent study in patients with diffuse large B-cell lymphomas, reported that an increase of CD163+ (M2) cells predicts poor prognosis, while an increase in CD68+ cells was related to improved overall survival (94). There is still a lot of work to clarify the role of TAM in cancer microenvironment, as TAM infiltration does not appear to have the same prognostic value for different cancer type.

B) *Natural Killer (NK)*

Natural killer cells have been initially identified as a lymphoid population able to lyse MHC class I (MHC-I) negative tumor and virus-infected cells and to orchestrate innate immunity of the organism. The majorities of NK cells are localized in peripheral blood, lymph nodes, spleen, and bone marrow but can migrate toward inflammation sites, such as primary or metastatic cancer cells (95, 96). The fact that NK cells constitutively express lytic machinery able to kill target cells independently from any previous activation suggests a role for NK cells in the control of tumor growth and metastasis diffusion *in vivo*. Some of the mechanisms are aborted below.

NK cells respond to the absence of class I MHC. Some tumors lose expression of class I MHC molecules, maybe as a result of selection against CTL. This loss of class I MHC molecules by tumors make them particularly good targets for NK cells. In addition NK cells can be targeted to IgG antibody coated cells by Fc receptors (CD16). The tumoricidal capacity of NK cells is increased by cytokines including interferon and interleukins. (97-99).

There are clinical and experimental evidence demonstrating the implication of NK cells in tumor immunity. Over the last few years, studies have revealed the phenotypic status and functionality of NK cells in tumor site and also in peripheral blood of cancer patients. First studies on tumor immune infiltration have shown that the presence of NK cells represents a positive prognostic marker in different colorectal, gastric carcinomas and squamous cell lung cancer (100-102).

C) *Dendritic Cells (DCs)*

DCs are potent professional antigen presenting cells, and bridge between innate and adaptive immune system. The two major groups of DC are known as the myeloid DC and the plasmacytoid DC. Functional subsets of myeloid DC in the skin, epidermal Langerhans cells and dermal interstitial DC are also characterized with distinct immune induction potentials. Activated epidermal

Langerhans cells secrete interleukin 15 (IL-15) and induce CD4⁺ and CD8⁺ T cell priming to elicit cellular immunity. Dermal interstitial DC stimulates B cell priming to produce humoral immunity (103, 104). Engaging DC via different receptors and subpopulations may stimulate different inflammation responses, producing multiple T cell outcomes including T helper cells of type 1 (Th1), Th2, Th17, Th21 and T regulatory cells.

In tumor microenvironment, DCs will phagocytose apoptotic cancer cells; a reaction mediated by the interaction of $\alpha\beta 5$ integrin (expressed by tumor cells) with CD36 receptors (expressed by DC) (105). Similar to macrophages, DC can recognize the so-called “eat me” signals expressed on apoptotic cells through endocytotic receptors, scavenger receptors, and TIM receptors. Phagocytosis of apoptotic tumor cells by DC in the absence of danger signals generally leads to immune tolerance (106).

Antibody-coated tumor cells are internalized into DC through activating Fc γ R. This internalization leads to more efficient MHC class I and II-restricted antigen presentation and induces tumor-specific effector and memory T cells (107).

2.2.2 Adaptive immune response

Unlike the innate immunity, the adaptive immune system is highly specific to a particular antigen. Adaptive immunity can also provide long-lasting protection, by the formation of a memory response. The adaptive immune response destroys invading pathogens, but can also be directed toward infected cells or defective cells like for cancer cells. Tumor cells express antigens that are recognized as foreign by the adaptive immune system, either by T or B lymphocytes. Depending on the lymphocytic population that recognize the antigen different adaptive immune response is initiated: —antibody responses and cell-mediated immune response—are also carried by two different lymphocytes (respectively B cells and T cells) (81).

Even if the antibody response is important in the context of immunosurveillance of the tumors, we will focus mainly on the cell-mediated immune response.

A) T lymphocytes responses to tumor

Tumor associated antigens (TAAs) are expressed by tumor cells. These cells can be phagocytized by antigens presenting cells (mainly macrophages and DCs). APCs present these antigens with their class II MHC molecules to T cell receptor (TCR) expressed at the surface of T lymphocytes. (108).

TAA can also be presented by cancer cells with class I MHC molecules and can be directly recognized by the TCR of T lymphocytes(108).

Each T cell has a unique TCR that respond to a unique antigen. The principal TAAs found in cancer are recapitulated in table 2.2.

Classification of tumour antigen	Mechanism of immune activation	Example
Cancer-testis antigens	Normal expression found in spermatocytes in testis (occasionally placenta), which is an immune-privileged site. Therefore, expression elsewhere in the body triggers T cell activation.	MAGE (melanoma antigen) BAGE (B antigen) GAGE (G antigen)
Differentiation antigens	Antigen is expressed by the tumor and the normal tissue from which it arose.	CEA – expressed in embryonic tissue and over-expressed in colorectal cancer. Gp100 – expressed in melanocytes and melanoma. PSA – expressed in normal prostate and over-expressed in prostate cancer.
Over-expressed tumourassociated antigens	Level of expression in normal tissue is below the threshold for T cell activation. Over- expression by malignant cells overrides tolerance and triggers T cell activation.	Her2 – over-expressed in breast cancer. AFP – over-expressed in hepatocellular cancer and certain germ cell tumours.
Tumour-specific antigens	These arise from genetic mutations or splicing aberrations, generating a protein that is foreign to the host immune system.	Mutant K-RAS in colorectal cancer.
Fusion proteins	Chromosomal translocation in certain tumours results in fusion of distant genes and expression of an abnormal fusion protein that is foreign to the host immune system.	BCR-ABL in CML and some ALL. EML4-ALK in non-small cell lung cancer.

Table 2.2: Classification of tumor-associated antigens that are recognized by T cells (109).

The recognition of a specific antigen by lymphocytes leads to a clonal expansion and proliferation of the lymphocyte that depend on the strength of the signal. Figure 2.5 recapitulates this clonal expansion that lead to anergic T cells (if the signal is too weak) or effector T cells and memory T cells.

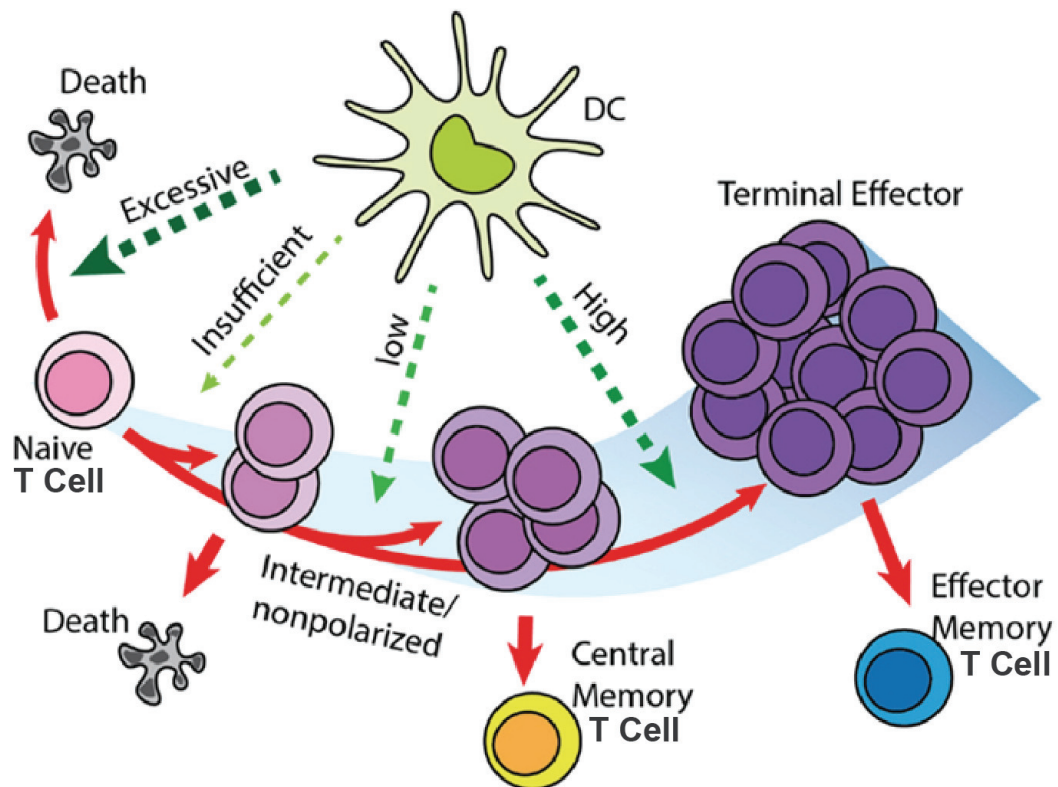


Figure 2.5: “Signal-strength model” of T cell differentiation. T cells receive antigenic, co-stimulatory, and inflammatory signals from DC during priming. In concert, these of stimulations determine the fate of T cell clonal expansion and differentiation. Greater proliferation correlates with stronger signal. However, insufficient stimulation results in death by neglect, while excessive stimulation causes activation induced cell death. Stronger stimulation also drives T cells toward terminal differentiation and reduces their memory-forming potential (110).

The biology of T cells is very complex. There are 3 main subtype of T lymphocytes population important for the comprehension of the adaptive immune response toward cancer:

1) CD4+ helper T lymphocyte (Th)

CD4 T lymphocytes play a central role in orchestrating the beginning and maintenance of the adaptive immune response. More than 30 years ago

Mitchison et al. recognized the ability of CD4 T cells to help antibody responses (111). The demonstration that CD4 T cell help was required for another specialized function of the immune system was the first example of cooperation between two cell populations in producing an immunological effect. A few years later it was also demonstrated that CD4⁺ T cells also help the activation and expansion of CD8 T cells (112). CD4 T cells can be divided into two main categories: T helper 1 (Th1) and T helper 2 (Th2) depending on the cytokines they produce in response to antigen activation. Th1 cells produce IL-2, IL-12 and IFN- γ . Th2 produce IL-4 and IL-5. This functional/phenotypic dichotomy influences the production of antibodies of the different isotypes and the types of CD8 T cells that originate after activation by antigen (113, 114).

In cancer it has been shown that Th1 promote CD8⁺ T cells expansion, upregulation of the MHC class I at the surface of tumor cells. Presence of Th1 in the tumor microenvironment is considered good prognostic in the majority of cancer studies (115, 116).

T_H2 cells contribute to the development of M2 macrophages through the production of the cytokine IL-10 and IL-4 and seem to be associated with aggressive tumors (116). However, this is not a general phenomenon, as T_H2 cells are also associated with favorable outcome in Hodgkin's lymphoma and breast cancer which suggests a protective effect of antibodies in these diseases(117, 118).

2) CD8⁺ cytotoxic T lymphocyte (CTL)

Cytotoxic CD8⁺ T lymphocytes are the keystone of the cancer elimination process. They are crucial components of tumor-specific cellular adaptive immunity. They directly attack tumor cells presenting tumor-associated antigen peptide with MHC class I on their surface. Activated CTL release perforin and granzyme that lead to the formation of pore within the cytoplasmic membrane and activate the caspase pathway of the target cell (119) (fig 2.6).

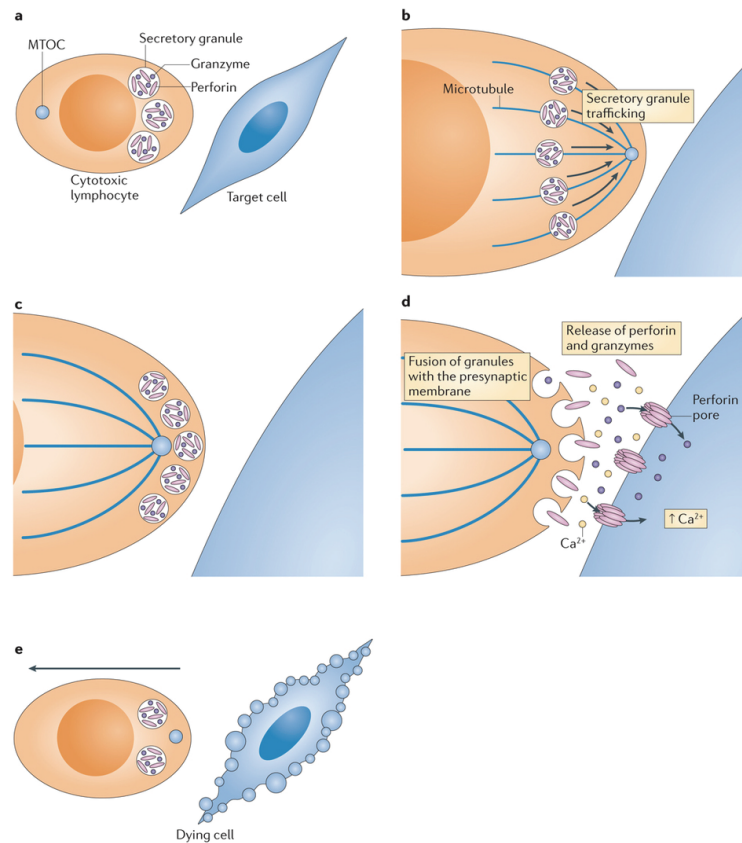


Figure 2.6: Granzyme/Perforin mediated cytotoxic action of CTL. The cytotoxic lymphocyte recognizes its target cell and forms an immunological synapse (part **a**). The microtubule-organizing center (MTOC) of the cytotoxic lymphocyte polarizes and secretory granules traffic towards the presynaptic membrane (part **b**). The secretory granules then fuse with the presynaptic membrane and release performing and granzymes into the synaptic cleft (parts **c** and **d**). At the postsynaptic membrane, perforin forms large transmembrane pores that enable the diffusion of granzymes into the target cell cytosol. Granzymes then initiate apoptosis of the target cell, and the cytotoxic lymphocyte detaches from the dying cell (indicated by the arrow; part **e**) and can interact with another target cell to carry out serial killing (not shown). (119)

CD8+ T cells produce $\text{INF}\gamma$ following interaction with their tumor targets. The $\text{INF}\gamma$ dependent mechanisms of tumor cell cytostasis and killing consequently occur by cell cycle inhibition, apoptosis, angiostasis, and induction of macrophage tumoricidal activity(120, 121).

Immunohistochemical studies have concluded that tumor infiltrating CD8+ lymphocytes have antitumor activity as judged by their favorable effect on patients' survival in colorectal, ovarian esophageal, renal, lung and pancreatic tumors (122-129).

3) CD4+CD25+FoxP3+ Regulatory T lymphocyte (Treg)

Among T cells recruited to human solid tumors are CD4+FOXP3+CD25+ regulatory T cells (Treg). These T cells accumulate in tumors and in the peripheral blood of patients with cancer (130). The increased Treg frequency has been generally considered as a marker of poor prognosis in cancer presumably due to Treg-mediated suppression of anti-tumor immunity, which benefits the tumor (131-133). However, several lines of evidence suggest that Treg might play a dual role in carcinogenesis. On the one hand, Treg can down-regulate inflammation, which in some cancers has been linked to tumor progression (134) and thus Treg benefit the host. On the other hand, Treg accumulations in cancer predict poor outcome (131-133). It appears that Treg can benefit the host by controlling inflammation but can also be harmful by suppressing anti-tumor immunity and promoting cancer progression.

As mentioned above, the function of lymphocytes in cancer is very variable between subtypes. Figures 2.7 resume the prognostic factors of all subtypes of population in 124 published articles.

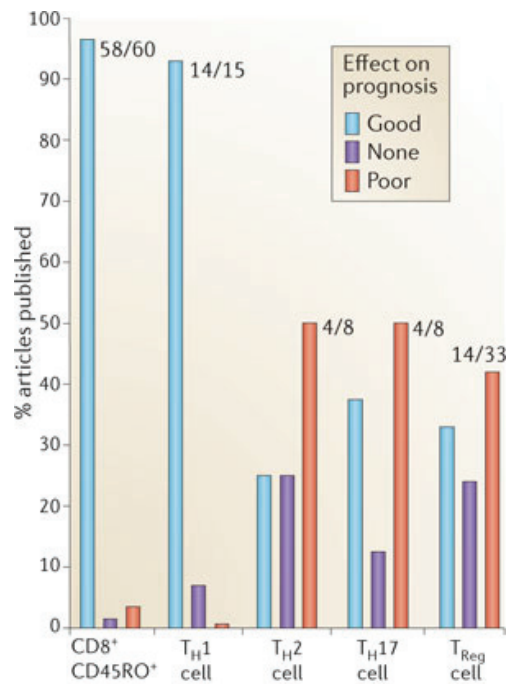


Figure 2.7: The prognostic impact of T cells in tumor. The analysis of 124 published articles studying the impact of cytotoxic T cells, memory T cells, regulatory T (T_{Reg}) cells and T helper (T_H) cell subpopulations with regard to prognosis of cancer patients (20 different cancer types were analyzed) is represented. 'Good' means that the cell type is associated with a good prognosis, 'none' means that there was no correlation and 'poor' means that the cells are associated with a poor prognosis (116).

2.3 Principles of immunosurveillance and immunoediting

The idea that the immune system, might recognize and destroy tumor cells emerged more than 50 years ago (135). First controversial, for the concept of cancer immunosurveillance (136), this aspect of tumor immunology regained interest in the mid-1990s with the observations that transplanted tumors grew more robustly in mice treated with INF- γ neutralizing monoclonal antibodies (137) and that immunodeficient mice lacking either IFN- γ responsiveness (IFNGR1, a component of the IFN- γ receptor) or an intact T cell compartment were more susceptible to methylcholanthrene (MCA)-induced sarcoma formation (138, 139).

Over the past decade, studies performed by different teams validated the concept of cancer immunosurveillance, demonstrating unequivocally that the

immune system can indeed protect mice from outgrowth of many different types of primary and transplantable tumors (138, 140, 141). An important study reported in 2001 provided evidence that the immune system controlled not only tumor quantity but also tumor quality (i.e., immunogenicity) (140). In this study, immunodeficient mice lacking either IFN- γ responsiveness (IFNGR1) or recombination activating gene-2 (RAG-2) were more prone to develop spontaneous neoplasia upon aging and were more susceptible to MCA -induced sarcomas compared with wild-type mice. In addition, 40% of MCA-induced sarcomas derived from immunodeficient *Rag2*^{-/-} mice were spontaneously rejected when transplanted into naive syngeneic wild-type mice, whereas all MCA sarcomas derived from immunocompetent wild-type mice grew progressively when transplanted into naive syngeneic wild-type hosts (140). Thus, tumors formed in the absence of an intact immune system are, more immunogenic than tumors that arise in immunocompetent hosts. These results show that the immune system not only protects the host against tumor formation, but also edits tumor immunogenicity. These new data prompted a refinement of the cancer immunosurveillance concept and led to the formulation of the cancer immunoediting hypothesis, which stresses the dual host-protective and tumor-sculpting actions of immunity on developing tumors.

In cancer immunoediting all the immune populations that infiltrate tumors are involved and interact with tumor cells. This complex process can be separate into three phases (figure 2.8). That will be presented in the coming sections.

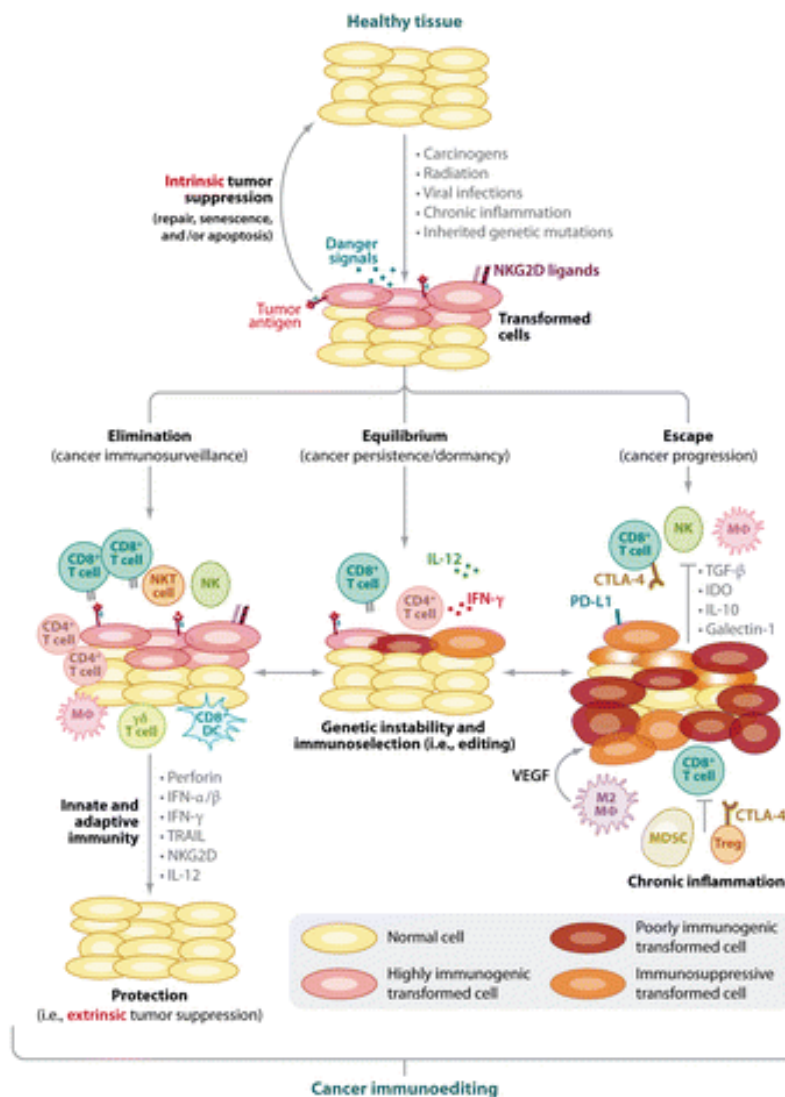


Figure 2.8: The three phases of cancer immunoediting. Cancer immunoediting is the result of three processes that function either independently or in sequence. Once normal cells are transformed into tumor cells by the combination of acquired oncogenes and failed intrinsic tumor-suppressor mechanisms, the immune system may function as an extrinsic tumor suppressor by eliminating tumor cells or preventing their outgrowth. In the first phase, elimination, previously known as cancer immunosurveillance, innate and adaptive immune cells and molecules recognize transformed cells and destroy them, resulting in a return to normal physiological tissue. However, if antitumor immunity is unable to completely eliminate transformed cells, surviving tumor variants may enter into the equilibrium phase, where cells and molecules of adaptive immunity prevent tumor outgrowth. These variants may eventually acquire further mutations that result in the evasion of tumor cell recognition, killing, or control by immune cells and progress to clinically detectable malignancies in the escape phase. (Abbreviations: CTLA-4, cytotoxic T lymphocyte associated protein-4; IDO, indoleamine 2,3-deoxygenase; IFN, interferon; IL, interleukin; Mφ, macrophage; MDSC, myeloid-derived suppressor cells; NK, natural killer; NKG2D, NK group 2, member D; PD-L1, programmed cell death 1 ligand 1; TGF-β, transforming growth factor-β; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.) (142)

2.3.1 Elimination phase: Cancer immunosurveillance.

The elimination phase represents the original concept of cancer immunosurveillance (Figure 2.8 left; Figure 2.9). If this phase successfully eradicates the developing tumor, it represents the complete immunoediting process without progression to the subsequent phases. Some of immune components involved in this phase are identified, but their precise roles need to be clarified and other participants identified.

The immune system being an extrinsic tumor suppressor mechanism, it is envisioned that it enrolls in the immunosurveillance only after transformed cells have circumvented their intrinsic tumor-suppressor mechanisms (143). Immunologic rejection of a developing tumor, likely requires an integrated response involving both the innate and adaptive arms of the immune system (section 2.2).

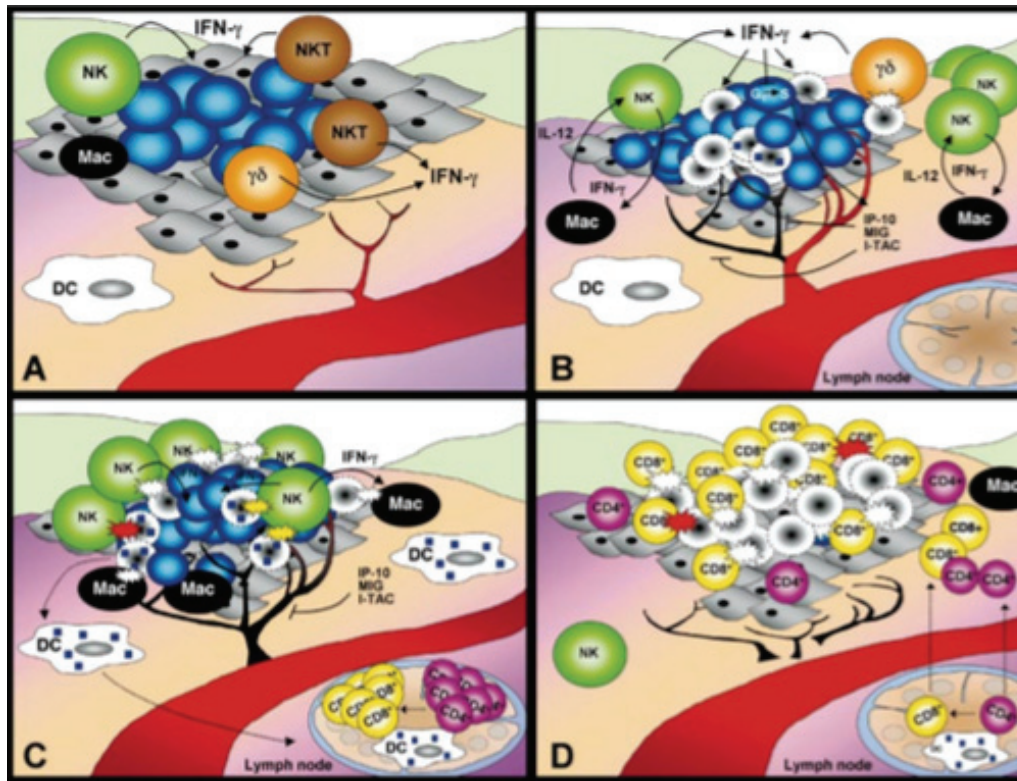


Figure 2.9: A proposed model for the elimination phase of the cancer immunoediting process. The events underlying this process are described in the text. Tumor cells are in blue; non-transformed cells in gray; lymphocytes, dendritic cells (DC), and macrophages (Mac) are marked and colored appropriately. Dead tumor cells are identified as white to gray gradient circles surrounded by a dashed black line, and tumor antigens are in blue squares. Panel (a) represents the initiation of the response, wherein cells of innate immunity recognize the nascent tumor. In panel (b), the initial amount of IFN- γ produced starts a cascade of innate immune reactions that result in some tumor cell death by both immunologic and non-immunologic mechanisms. In panel (c), events of innate immunity charge the adaptive response; tumor cells killed due to the increased cytotoxic activities of NK cells and activated macrophages are ingested by DCs, which migrate to the draining lymph node and present antigen to naive CD4⁺ and CD8⁺ T cells. In panel (d), tumor-specific CD4⁺ and CD8⁺ T cells home to the tumor along a chemokine gradient where they recognize and destroy tumor cells expressing distinctive tumor antigens (136).

A) Initiation

Initiation of the antitumor immune response (Figure 2.9a) occurs when proinflammatory molecules and cytokines, produced by tumor cells themselves, are detected by cells of the innate immunity (144-146). Once recruited to the developing tumor mass, NK cells, and/or macrophages will recognize molecules, such as NKG2D ligand which expression is induced on tumor cells inflammation

or the cellular transformation. This recognition leads to the production of IFN- γ by innate immunity effectors.

B) Second step

In the second step (Figure 2.9b), the effects of innate immune recognition of the tumor are amplified. The IFN γ released at the tumor site by innate immunity effectors, induces a local production of chemokines that in turn, recruit effectors of the innate immune response to the tumor site.

Products generated during remodelling of the extracellular matrix may induce tumor-infiltrating macrophages to produce low amounts of IL-12 (147). That stimulates tumor-infiltrating NK cells to produce low amounts of IFN- γ , which in turn activate macrophages in the tumor to produce more IL-12, leading to increased IFN- γ production by NK cells. In addition to this positive feedback system, the binding of NK cell-activating receptors to their cognate ligands on tumor cells stimulates even more NK cell IFN- γ production that can now activate a number of IFN- γ -dependent processes including anti-proliferative, pro-apoptotic, and angiostatic (148-153) effects that result in the killing of a proportion of the tumor. In addition, macrophages activated by IFN γ will express tumoricidal products such as reactive oxygen and reactive nitrogen intermediates (154) and activated NK cells can kill tumor cells via TRAIL- or perforin-dependent mechanisms (155, 156). At the end of this step tumor antigens are liberated from dead tumor cells and the adaptive immune system will be recruited.

C) Third step

In the third step (Figure 2.9c), tumor antigens liberated by the effects of innate immunity on the tumor drive the development of tumor-specific adaptive immune responses. Immature dendritic cells (DCs) that have been recruited to the tumor site become activated either by exposure to the cytokine milieu created during the ongoing attack on the tumor by innate immunity or by interacting with tumor-infiltrating NK cells (157). The activated DCs can acquire tumor antigens directly

by ingestion of tumor cell debris or potentially through indirect mechanisms involving transfer of tumor cell–derived heat shock protein/tumor antigen complexes to DCs (158). Activated, antigen-bearing mature DCs then migrate to the draining lymph node (159), where they induce the activation of naive tumor-specific Th1 CD4⁺ T cells. Th1 cells facilitate the development of tumor-specific CD8⁺ CTL induced via cross-presentation of antigenic tumor peptides on DC MHC class I molecules (160).

D) Final step

In the fourth step (Figure 2.9d), the development of tumor-specific adaptive immunity provides the host with a capacity to completely eliminate the developing tumor. Tumor-specific CD4⁺ and CD8⁺ T cells home to the tumor site, where they participate in the killing of antigen-positive tumor cells. CD4⁺ T cells produce IL-2 that, together with host cell production of IL-15, helps to maintain the function and viability of the tumor-specific CD8⁺ T cells. Tumor-specific CD8⁺ T cells will efficiently recognize their targets (owing to the enhanced immunogenicity of tumor cells that have been exposed to the IFN- γ produced in steps 1 and 2 (140)) and will induce tumor cell death by both direct and indirect mechanisms. It is likely that these CD8⁺ T cells directly kill many of the tumor cells *in vivo*. However, these cells will also produce large amounts of IFN- γ following interaction with their tumor targets and thus should also induce tumor cell cytostasis and killing by the IFN- γ -dependent mechanisms of cell cycle inhibition, apoptosis, angiostasis, and induction of macrophage tumoricidal activity. These two scenarios are not mutually exclusive and most likely occur concomitantly; however, their relative contributions may vary among different tumors. Thus, the elimination phase of cancer immunoediting is a continuous process that must be repeated each time antigenically distinct neoplastic cells arise. For this reason, it is particularly noteworthy that cancer is more prevalent in aged populations where immune system function, and therefore cancer immunosurveillance, begins to decline.

2.3.2 Equilibrium phase: Immune mediated tumor dormancy

In the equilibrium phase (Figure 2.8 middle), the host immune system and any tumor cell variant that has survived the elimination phase enter into a dynamic equilibrium, wherein lymphocytes and IFN γ exert potent and relentless selection pressure on the tumor cells that is enough to contain, but not fully extinguish, a tumor bed containing many genetically unstable and mutating tumor cells. Although many of the original tumor cell escape variants are destroyed, new variants arise carrying different mutations that provide them with increased resistance to immune attack. The end result of the equilibrium process is a new population of tumor clones with reduced immunogenicity, hewn from a heterogeneous parental population by the sculpting forces of the immune system.

Equilibrium is probably the longest of the three phases and may occur over a period of many years in humans. Indeed, it has been estimated that for many solid human tumors there can be a 20-year interval between initial carcinogen exposure and clinical detection of the tumor (161). During this period, the heterogeneity and genetic instability of cancer cells that survive the elimination phase are possibly the principal forces that enable tumor cells to eventually resist the host's immunological siege. It has been proposed that the "mutator phenotype" of tumor cells (161) may result from the three types of genetic instability observed in cancer: nucleotide-excision repair instability (NIN), microsatellite instability (MIN), and chromosomal instability (CIN) (162). Of the three, CIN is thought to be the predominant mechanism responsible for destabilizing genomic integrity, and the observation that cancer cell genomes display gains or losses of whole chromosomes (i.e., aneuploidy) associated with an estimated loss of 25%–50% of their alleles reflects the degree of genomic upheaval associated with the CIN phenotype (162). Clearly, genomic instability has the potential to spawn tumor variants of reduced immunogenicity, and some of these will display an enhanced capacity to grow in an unfettered immune selecting environment. A complete mechanistic understanding of the equilibrium

phase will require the development of new tumor models to better define the cell-intrinsic mechanisms that generate new tumor phenotypes and to identify the tumor-sculpting immune “editors.”

2.3.3 Escape phase: Failure of cancer immunosurveillance

In the escape phase (Figure 2.8 right), tumor cell variants selected in the equilibrium phase now can grow in an immunologically intact environment. This breach of the host’s immune defenses most likely occurs when genetic and epigenetic changes in the tumor cell confer resistance to immune detection and/or elimination, allowing the tumors to expand and become clinically detectable. Because both the adaptive and innate compartments of the immune system function in the cancer immunosurveillance network, tumors most likely would have to circumvent either one or both arms of immunity in order to achieve progressive growth. Individual tumor cells may employ multiple immunoevasive strategies to elude the powerful integrated innate and adaptive antitumor immune responses to their immunogenic progenitors. Thus, it is likely that several distinct immunologically driven tumor-sculpting events must occur before the final immunogenic phenotype of a malignant cell is ultimately established (142).

Over the years, multiple strategies of the tumor cells to evade the immune response have been discovered. Since every strategies are very complex on their own, we focus on immune checkpoint upregulation as they are generating tremendous enthusiasms for immunotherapeutic approaches.

A) Immune checkpoint upregulation in cancer

The myriad of genetic and epigenetic alterations that are characteristic of all cancers provide a diverse set of antigens that the immune system can use to distinguish tumor cells from their normal counterparts. In the case of T cells, the ultimate amplitude and quality of the response, which is initiated through antigen recognition by the T cell receptor (TCR), is regulated by a balance between co-stimulatory and inhibitory signals (that is, immune checkpoints) (163, 164) (Figure 2.8). Under normal physiological conditions, immune checkpoints are crucial for the maintenance of self-tolerance (that is, the prevention of autoimmunity) and also to protect tissues from damage when the immune system is responding to pathogenic infection. The expression of immune-checkpoint proteins can be deregulated by tumors as an important immune resistance mechanism.

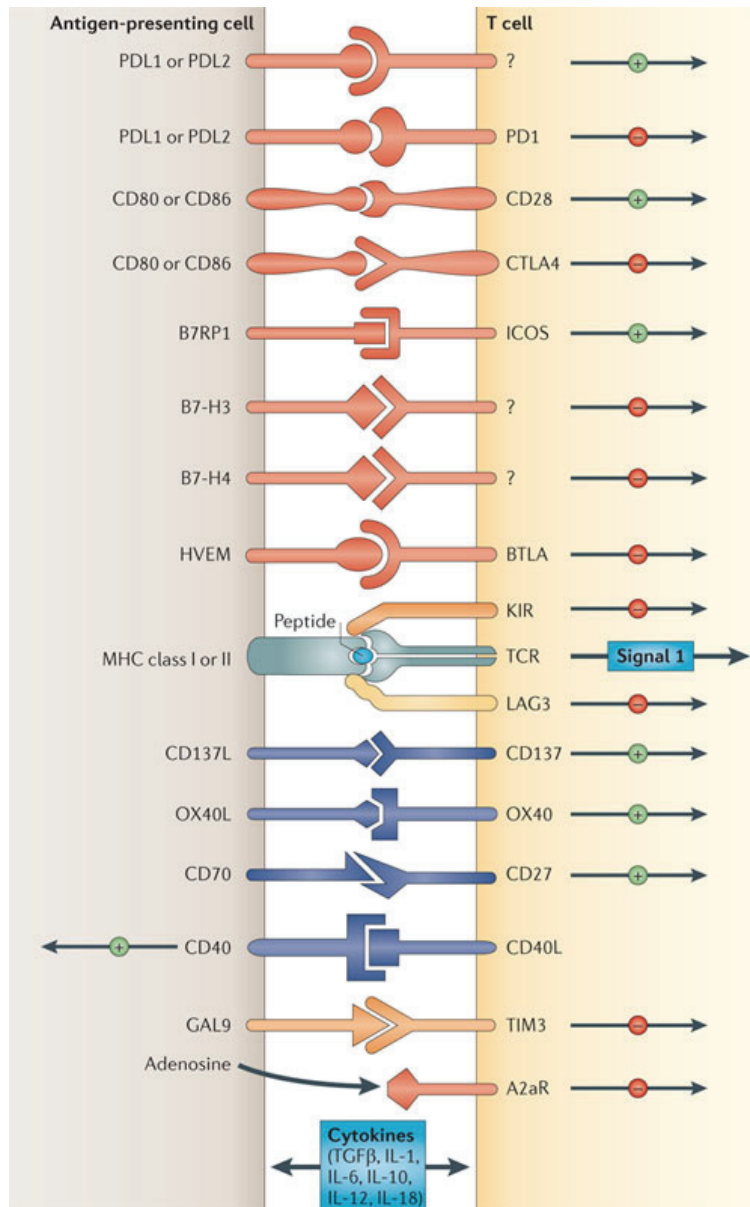


Figure 2.10: Co-stimulatory and inhibitory signal of TCR. T cell response to antigen (which is mediated by peptide–major histocompatibility complex (MHC) molecule complexes that are recognized by the T cell receptor (TCR)). These responses can occur at the initiation of T cell responses in lymph nodes (where the major APCs are dendritic cells) or in peripheral tissues or tumors (where effector responses are regulated). In general, T cells do not respond to these ligand–receptor interactions unless they first recognize their cognate antigen through the TCR. Many of the ligands bind to multiple receptors, some of which deliver co-stimulatory signals and others deliver inhibitory signals. In general, pairs of co-stimulatory–inhibitory receptors that bind the same ligand or ligands such as CD28 and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) display distinct kinetics of expression with the co-stimulatory receptor expressed on naive and resting T cells, but the inhibitory receptor is commonly upregulated after T cell activation. One important family of membrane-bound ligands that bind both co-stimulatory and inhibitory receptors is the B7 family (165).

As shown in figure 2.10 there is a multitude of checkpoint inhibitor that regulates the quality of the TCR induction. Among them, two were the object of a multitude of study on cancer research: CTLA-4 and PD-1.

1) CTLA-4

CTLA-4, the first immune-checkpoint receptor to be clinically targeted, is expressed exclusively on T cells where it primarily regulates the amplitude of the early stages of T cell activation. Primarily, CTLA-4 counteracts the activity of the T cell co-stimulatory receptor, CD28 (166, 167). CD28 and CTLA-4 share identical ligands: CD80 (also known as B7.1) and CD86 (also known as B7.2) (168). Although the exact mechanisms of CTLA-4 action are under considerable debate, it has been proposed that its expression on the surface of T cells dampens the activation of T cells by outcompeting CD28 in binding CD80 and CD86, as well as actively delivering inhibitory signals to the T cell (169). The specific signalling pathways by which CTLA-4 blocks T cell activation are still under investigation, although a number of studies suggest that activation of the protein phosphatases, SHP2 (also known as PTPN11) and PP2A, are important in counteracting kinase signals that are induced by TCR and CD28 {Rudd, 2009 #4115}. Moreover, CTLA-4 also confers 'signaling-independent' T cell inhibition through the sequestration of CD80 and CD86 from CD28 engagement, as well as active removal of CD80 and CD86 from the antigen-presenting cell (APC) surface (170). The central role of CTLA-4 in inactivation in T cell is demonstrated by the lethal systemic immune hyperactivation phenotype of *Ctla4*-knockout mice (171).

Even though CTLA-4 is expressed by activated CD8⁺ effector T cells, the major physiological role of CTLA-4 seems to be through distinct effects on the two major subsets of CD4⁺ T cells: downmodulation of helper T cell activity and enhancement of regulatory T (Treg) cell immunosuppressive activity (172, 173). CTLA-4 blockade results in a broad enhancement of immune responses that are dependent on helper T cells and, conversely, CTLA-4 engagement on T_{Reg} cells

enhances their suppressive function. *CTLA-4* is a target gene of the forkhead transcription factor FOXP3 (174), the expression of which determines the T_{Reg} cell lineage (175), and T_{Reg} cells therefore express CTLA-4 constitutively. Although the mechanism by which CTLA-4 enhances the immunosuppressive function of T_{Reg} cells is not known, T_{Reg} cell-specific CTLA-4 knockout or blockade significantly inhibits their ability to regulate both autoimmunity and antitumor immunity(172, 173)(Figure 2.11a).

2) PD-1

In contrast to CTLA-4, the major role of PD-1 is to limit the activity of T cells in peripheral tissues at the time of an inflammatory response to infection and to limit autoimmunity (176, 177) (Figure 2.11b). This translates into a major immune resistance mechanism within the tumor microenvironment (178). PD-1 expression is induced when T cells become activated (179). When engaged by one of its ligands, PD-1 inhibits kinases that are involved in T cell activation through the phosphatase SHP2, although additional signaling pathways are also probably induced (177). Also, because PD-1 engagement inhibits the TCR 'stop signal', this pathway could modify the duration of T cell–APC or T cell–target cell contact(180). PD-1 is expressed on a large proportion of tumor-infiltrating lymphocytes (TILs) from many different tumor types (181).

The two ligands for PD-1 are PD-1 ligand 1 (PD-L1; also known as B7-H1 and CD274) and PD-L2 (also known as B7-DC or CD273) (177, 182). Just as PD-1 is highly expressed on TILs in cancers, the PD1 ligands are commonly upregulated on the tumor cell surface from many different human tumors (164, 178). On cells from solid tumors, the major PD-1 ligand that is expressed is PD-L1. Forced expression of PD-L1 on mouse tumor cells inhibits local antitumor T cell-mediated responses (178).

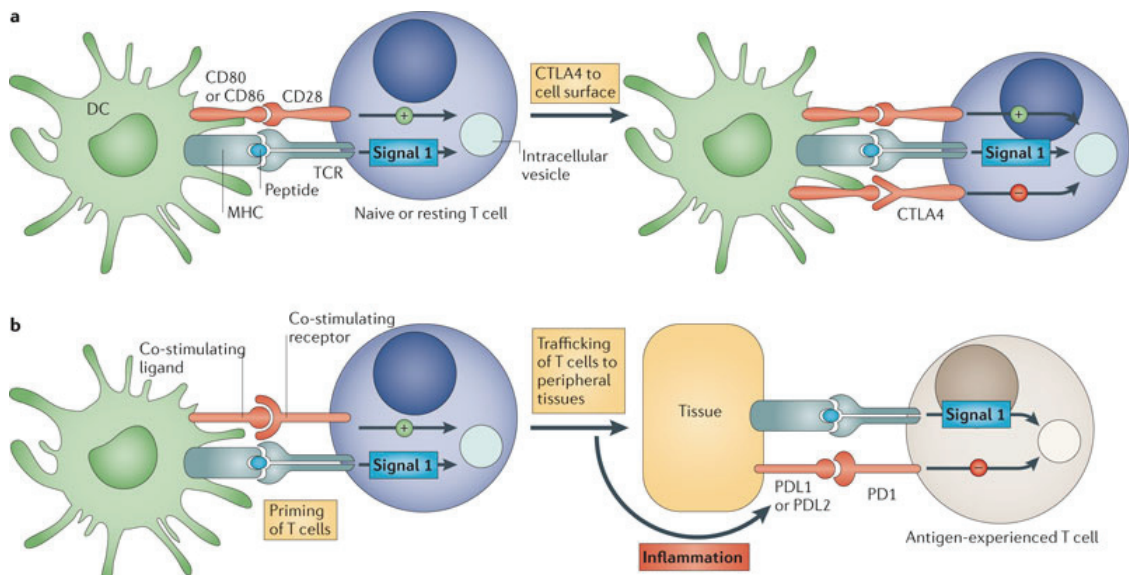


Figure 2.11: Immune checkpoint in cancer. a | The cytotoxic T-lymphocyte-associated antigen 4 (CTLA4)-mediated immune checkpoint is induced in T cells at the time of their initial response to antigen. The level of CTLA4 induction depends on the amplitude of the initial T cell receptor (TCR)-mediated signaling. High-affinity ligands induce higher levels of CTLA4, which dampens the amplitude of the initial response. The key to the regulation of T cell activation levels by the CD28–CTLA4 system is the timing of surface expression. CTLA4 functions as a signal dampener to maintain a consistent level of T cell activation in the face of widely varying concentrations and affinities of ligand for the TCR. **b** | By contrast, the major role of the programmed cell death protein 1 (PD-1) pathway is not at the initial T cell activation stage but rather to regulate inflammatory responses in tissues by effector T cells recognizing antigen in peripheral tissues. Activated T cells upregulate PD-1 and continue to express it in tissues. Inflammatory signals in the tissues induce the expression of PD-1 ligands, which downregulate the activity of T cells and thus limit collateral tissue damage in response to a microorganism infection in that tissue. The best characterized signal for PD-1 ligand 1 (PD-L1; also known as B7-H1) induction is interferon- γ (IFN γ), which is predominantly produced by T helper 1 (T_H1) cells, although many of the signals have not yet been defined completely. Excessive induction of PD-1 on T cells in the setting of chronic antigen exposure can induce an exhausted or anergic state in T cells. MHC, major histocompatibility complex (165).

The evident role of immune systems in restraining and/or promoting cancer growth makes it a target of choice for novel therapies. The next chapter will focus on these therapies now developed for the treatment of sarcomas.

3. Immunotherapies in sarcoma

Sarcomas have long been suspected to have a connection to the immune system. In 1891, physicians such as William Coley noted a relationship between infection and regression of sarcoma (183). Additionally, some patients with metastatic sarcomas can be cured with aggressive resection of all visible disease, unlike many other solid tumors. It is thought that immune surveillance and suppression of growth must play a role in the prevention of recurrence in these patients (184). Some sarcomas express highly immunogenic antigens, such as cancer testis antigens, microphthalmia transcription factor, and even the fusion proteins themselves. The presence of these antigens shows that immunotherapies could be operational for sarcomas treatments (table 2.3) (185, 186).

Over the past several years, immunotherapy has regained significant attention in solid tumors, among which sarcoma, even for tumors largely believed for having low immunogenicity, and novel targets and immunotherapeutic strategies are rapidly emerging. Here we review the different immunotherapies explored in sarcomas and chondrosarcomas and the future perspectives envisaged.

3.1 Cytokines therapies

Cytokines, including interleukins and growth factors, play an important role in the regulation of the immune system (187). Several studies have indicated that cytokine therapy could relieve immunosuppression in cancer patients (188) and therefore, cytokine therapy is a potential therapeutic approach for sarcoma patients. Two main cytokines therapies have been tested in sarcoma.

3.1.1 *IL-2*

Interleukin 2 (IL-2) stimulates T cells proliferation, induces generation of CTLs, and facilitates the maintenance of NK cells (189, 190). IL-2 is approved by the FDA for the treatment of metastatic renal cell carcinoma and melanoma, and responses to IL-2 have been reported in several other cancers including lung and breast cancers (191). High-dose IL-2 was given in relapsed pediatric patients

among which 4 patients with osteosarcoma and 2 patients with Ewing sarcoma. Two of the four osteosarcoma patients had complete responses, while the other two and both Ewing sarcoma patients had progressive disease (191). These results associated to the toxicity of high-dose IL-2 make the administration of IL-2 as a single agent unlikely in sarcoma. Several studies are ongoing in sarcomas combining IL-2 given in a variety of different routes and dosages with antibody therapy, vaccines, and adoptive cell therapy (192).

3.1.2 Interferon

Interferons are a complex family of molecules that bind to IFN receptors; IFN α and IFN β activate type I receptors, while IFN γ activates type II receptors (193). Both IFN α and IFN β activate immune cells and increase antigen presentation to T cells. IFN α is approved for use in melanoma and has also been studied in sarcomas. Interferons used in bones sarcomas are resume in table 3.1.

Study name	Patients	Type of sarcoma	Type of interferon	Dose/schedule	Outcome
Karolinska Hospital series	After surgical resection, 89 patients	Osteosarcoma	Interferon- α	Cohort 1 (70 patients) 3 × 10 ⁶ IU daily × 1 month, followed by 3×/week, 17 months Cohort 2 (19 patients) 3 × 10 ⁶ IU daily for 3–5 years	10-year metastatic free survival: 39% (95% CI 29–49%) 10-year sarcoma specific survival: 43% (95% CI 33–54%) Median time to metastasis 8 months (range 1–60 months) value not specified
COSS-80	100 patients after preoperative chemotherapy and surgical resection were randomized +/- interferon	Osteosarcoma	Interferon- β	100,000U/kg for 22 weeks (2 injections weekly × 2 weeks, daily × 4 weeks, weekly × 16 weeks)	30-month continuous disease-free survival: 77% interferon arm versus 73% noninterferon arm Log rank test
EURAMOS 1	715 patients that had good response to preoperative chemotherapy were randomized to postoperative chemotherapy +/- interferon	Osteosarcoma	Pegylated interferon- α 2b	0.5–1.0 μ g/kg/week for 2 years	Primary endpoint: event-free survival (EFS) 77% who received INT were disease free at 3 years compared to 74% Hazard ratio for EFS from adjusted Cox model was 0.82 (95% CI 0.61–1.11;) in favor of chemotherapy + interferon

Table 3.1: Interferons studies in bone sarcoma (192).

3.2 Vaccines therapies

Vaccines directed against specific tumor antigens were some of the earliest targeted immunotherapies tested. The aim of tumor vaccines is to induce an anti-tumor response through exposure to tumor antigens. Multiple clinical trials using vaccines that target whole cells, lysates, proteins, and peptides have been investigated in patients with sarcomas (Table 3.2) (194-196). In these studies, patients with chondrosarcoma show high expression of tumor-associated antigens (TAAs) and show good response to vaccine. However there were very few patients with chondrosarcoma in these studies and follow-up need to be done. Vaccines therapies have been validated for bones and soft tissues sarcoma. Table 3.3 review the main clinical studies made for sarcomas.

Histological subtype	Case no./ Cell line	SSX	NY-ESO-1	LAGE-1	MAGE-A1	MAGE-A3	MAGE-A4	MAGE-A10	CT-7	CT-10	SCP-1	GAGE	BAGE
Tumor samples													
Gastrointestinal stromal tumor	1	+	+++	+++	++	++	+	++	-	+	-	++	-
	2	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	+	-	-	-	+	-	-	+++	-	+	+
	4	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-
Synovial sarcoma	1	-	+++	++	+	+	+++	-	-	-	-	+	-
	2	-	+++	-	+	-	+++	-	-	-	-	-	-
Uterine leiomyosarcoma	1	+++	+	++	+	++	+	+	-	+	-	++	+
	2	-	-	-	-	-	-	-	-	-	-	++	-
	3	+++	-	+++	+++	+++	+++	+++	+++	+	-	+++	-
	4	+	+	-	+	+	-	-	-	+	-	++	+
	5	++	+++	-	-	++	+++	+++	-	-	-	++	-
Leiomyosarcoma	1	-	-	-	-	-	-	-	-	-	-	+	-
	2	-	-	-	-	-	++	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-
	4	++	-	-	+	-	++	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-
	6	+	-	++	-	++	-	-	+	++	-	+	-
	7	-	-	-	-	-	-	-	-	-	-	-	+
Angiosarcoma	1	-	+	-	-	+++	+++	-	-	-	-	-	-
	2	-	++	-	-	+	-	-	-	-	-	+	-
	3	-	-	-	-	++	-	-	-	-	-	-	-
Malignant fibrous histiocytoma	1	+	++	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	+++	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-
	4	+++	-	-	+	+++	-	+++	+	++	-	+++	+++
	5	-	-	-	-	-	-	-	-	-	-	+	-
Liposarcoma	1	+++	-	++	-	++	-	-	+	+++	-	+++	+++
	2	++	+++	+++	-	+	++	+	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	+++	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-
Osteosarcoma	1	+	-	-	-	-	-	-	-	-	+++	-	-
Chondrosarcoma	1	+++	+	+++	+++	+++	+++	-	+	++	-	+++	-
	2	+++	+	++	+	+	-	-	-	+	-	+	-
Tumor cell lines													
Ewing's sarcoma	SK-ES-1	-	-	-	-	++	-	-	-	-	+++	-	-
Leiomyosarcoma	SK-LMS-1	++	-	-	-	+	-	-	-	+++	-	++	-
Uterine leiomyosarcoma	SK-UT-1	-	-	-	-	-	-	-	-	-	-	-	-
Liposarcoma	SW 872	++	-	-	++	+++	++	+++	++	+++	-	+++	++
Osteosarcoma	Saos-2	++	+	+++	++	+++	-	-	-	+++	-	++	-
Osteosarcoma	HOS	-	-	-	-	-	-	-	-	-	-	+	-
Uterine sarcoma	MES-SA	-	-	-	-	++	-	-	-	-	-	-	+++
Fibrosarcoma	HT-1080	+++	+++	+++	++	+++	-	-	-	+++	-	+++	+++

Table 3.2: TAAs expression in sarcoma tumor samples and cell lines (194).

Vaccine	Sarcoma histology	Number of patients	Immune response	Results
Irradiated autologous tumor cells	Various pediatric	16	Not reported	16.6m versus 8.2m survival (skin test responders)
Dendritic cell pulsed with autologous tumor lysate	Various pediatric	10	Not reported	One response in patient with fibrosarcoma
Dendritic cell pulsed with peptides from tumor specific translocation breakpoints and E7	Metastatic Ewing's family of tumors or alveolar rhabdomyosarcoma	30/52 initiated vaccine after standard therapy	39% with immune response to translocation breakpoint 25% with E7-specific response	5-year OS: 31% for all patients versus 43% for patients initiating immunotherapy
105AD7 (CD55 target)	Osteosarcoma	28 patients within 1–6 months of chemotherapy	20/28 (71%) showed T cell proliferation response in vitro to 105AD7 9/28 (32%) weak antibody response to CD55	2 patients with possible clinical responses, alive and disease free 5.8 and 6.5 years from time of diagnosis
Dendritic vaccine pulsed with synthetic tumor specific peptide	Posttransplant, residual tumor (synovial, Ewing's)	5	DTH response against tumor lysate in 1 patient	1 patient complete response, 77 months
Peptide encompassing SYT-SSX	Synovial	6	Peptide specific CTLs generated in 4 patients	Suppression of tumor progression 1 patient
SYT-SSX derived peptide	Synovial	20	9 showed twofold increase in CTLs in tetramer analysis	1/9 stable disease (received vaccine with peptide alone) 6/12 stable disease (received vaccine with incomplete Freund's adjuvant)

Table 3.3: Vaccines immunotherapy in sarcomas (192)

3.3 Adoptive Cell Transfer

Adoptive cell transfer (ACT) means the transfer of immune cells with antitumor activity. This can be done by expansion and infusion of autologous or allogeneic tumor infiltrating lymphocytes, the use of genetically modified lymphocytes. Most recently scientist have generated chimeric antigen receptors (CAR) that will be explained below.

By using gamma retroviruses or lentiviruses, lymphocytes can be genetically modified to encode T cell receptors (TCR) that recognize specific tumor antigens or encode molecules that can enhance their antitumor activity (197). These lymphocytes then acquire antitumor activity.

Historically, these are conventional TCR that have alpha and beta chains that form heterodimers to recognize cancer antigens that are presented on the surface of MHC molecules on tumor cells (197). T cells genetically engineered to target NY-ESO-1 expressing synovial sarcoma have shown some promise (185). In a small study, patients with NY-ESO-1 expressing synovial sarcomas were treated with a lymphodepleting chemotherapy regimen consisting of cyclophosphamide and fludarabine, followed by infusions of autologous T lymphocytes designed to recognize a specific NY-ESO-1 antigen. Four of 6 patients with synovial sarcoma had evidence of partial response lasting from 5–18 months. Limitations of conventional TCR are that they are restricted to antigen presentation on specific MHC molecules (197). In addition, downregulation of class I MHC molecules has been described as a mechanism of avoiding TCR recognition (198).

3.4 Modulation of macrophage response in sarcoma

Mifamurtide, liposomal muramyl tripeptide phosphatidylethanolamine (L-MTP-PE), is a new agent that is a synthetic analog of a muramyl dipeptide (MDP) (199). Although its pharmacological behavior is similar to that of MDP, L-MTP-PE has a longer half-life than MDP (200). The intracellular pattern recognition molecule NOD2 detects MDP and enhances NF- κ B signaling (201). Therefore, recognition of L-MTP-PE by NOD2 stimulates the production of IL-1 β , IL-6, and TNF- α via the activation of NF- κ B signaling in monocytes and macrophages (202) and M2 to M1 conversion in macrophages (203).

The efficacy of L-MTP-PE treatment for osteosarcomas has been examined in dogs. Dogs with postoperative osteosarcomas were treated by intravenous L-MTP-PE injections. The median survival time of dogs treated by L-MTP-PE (222 days) was longer than that of non-treated dogs (77 days) (204). In human, intergroup study 0133 (INT 0133) began in 1993. 662 patients with osteosarcoma were recruited in this study. The aim of the study was to evaluate the efficacy of supplementation with ifosfamide (IFO) and L-MTP-PE in basic adjuvant chemotherapy (cisplatin, doxorubicin, and high-dose methotrexate (MAP)). Patients were randomly assigned to receive MAP alone, MAP + IFO, MAP + L-MTP-PE, and MAP + IFO + L-MTP-PE. It was observed that the addition of L-MTP-PE to chemotherapy improved the six-year overall survival rate from 70% to 78%. The hazard ratio for overall survival with the addition of MTP was 0.71 (95% CI: 0.52–0.96) (205). Therefore, L-MTP-PE has been approved in Europe for the treatment of osteosarcoma with chemotherapy. However, it has not been approved by FDA in the United States (202).

3.5 Immune Checkpoint Blockade

Recently, there has been much excitement about the potential of the immune checkpoint inhibitors in solid tumors including sarcomas following their clinical successes and approvals for treatment of metastatic melanoma and metastatic squamous non-small cell lung cancer(206-208).

3.5.1 CTLA-4 blockade

Ipilimumab is a human IgG4 monoclonal antibody that blocks the anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and was the first of the new generation of checkpoint inhibitors to gain FDA approval (206). CTLA-4 is a member of the immunoglobulin superfamily; after T cell activation, CTLA-4 is expressed on the plasma membrane of cells where it acts to inhibit T cell function through a variety of mechanisms, allowing tumor cells to escape immune surveillance (209, 210). The experience of ipilimumab in pediatric patients is

limited; GI toxicity was the major concern. A small phase II study in adults with synovial sarcoma had no clinical or immunological responses (207).

3.5.2 PD-1/PD-L1 blockade

Antibodies targeting the programmed cell death protein 1 pathway (PD-1/PD-L1) (nivolumab, pembrolizumab) function in a similar manner to ipilimumab by removing the brakes on T cells which then can perform active anti-tumor immune surveillance (209, 210). Preclinical studies have demonstrated expression of PD-1L in OS and suggest that high expression levels may correlate with worse clinical outcomes (211); *In vivo* studies using murine sarcoma models with anti-CTLA-4 And PD-1 antibodies have also shown promise for these agents (208). Currently, however, these agents have limited pediatric clinical data available; several trials with these agents for relapsed or refractory pediatric solid tumors are currently ongoing.

Despite the overall successes of checkpoint inhibitors, only subsets of patients with melanoma, lung cancer, ovarian cancer, NHL, and Hodgkin lymphoma have responded. Two important studies have examined the tumors of responders versus non-responders, one in melanoma and one in non-small-cell lung cancer (212, 213). In both cases, treatment efficacy was associated with a higher number of mutations in the tumors. In melanoma patients treated with ipilimumab, the investigators carefully examined the tumors of those who responded versus those who did not, and found that the responders had tumors with higher mutation rates and tumor antigens and in particular, those whose tumor neoantigens shared tetrapeptide sequences with viral antigens were most likely to be responders to checkpoint inhibition (213). To improve on the quality of response to immune checkpoint blockade, CTLA-4 and PD-1/PD-L1 antibodies are being tested in combination or when added to other anti-cancer agents such as chemotherapy, targeted therapy, radiotherapy, and other immunotherapy (209, 214, 215). Currently, the COG is conducting a phase I/II study (NCT02304458) of nivolumab alone or in combination with ipilimumab for relapsed and refractory solid tumors including sarcomas.

3.6 Futures directions for immunotherapies in sarcoma

Conventional treatment for bone and soft tissue sarcomas consists of surgical resection, chemotherapy, and radiotherapy. However, clinical outcomes by these therapeutic modalities have not significantly improved in recent decades. Under these circumstances, immunotherapy is expected to be a new therapeutic option for treatment. Cytokine therapies were initially regarded as a form of immunotherapy; however, their effectiveness was limited because of their toxicities. Only IFN- α -2 is used for maintenance therapy.

Although L-MTP-PE induces antitumor effects via macrophage activation, the FDA has not approved its use because of the limited effectiveness. In Europe, L-MTP-PE efficacy has been confirmed in an international multicenter study. Vaccine therapy using tumor lysates or lysate-derived DCs has been investigated only in small-scale studies and in non-sarcoma patients. CTA peptide and fusion protein peptide therapies are expected to be novel sarcoma-effective vaccines. Addition of L-MTP-PE as an adjuvant may improve the vaccine therapy outcome. Novel microparticle-based drug delivery systems, such as microemulsion, nanoemulsion, nanoparticles, liposomes, and others, can load many kinds of various drugs and improve the drug delivery to target sites (216-218). It has been reported that these systems improve the efficacy of vaccine and reduce adverse effects of cytokines (219, 220). Tuftsin, a tetrapeptide (Thr-Lys-Pro-Arg) fraction of immunoglobulin G molecule, binds to neutrophils and macrophages (221). Tuftsin stimulates their phagocytic activity and enhances expression of nitric oxide synthase in macrophages. It has been demonstrated that tuftsin improves the efficacy of antibiotics against protozoan, bacterial, and fungal infections. Besides, tuftsin-bearing liposomized etoposide enhanced the therapeutic efficacy in murine fibrosarcoma models (222).

Immune checkpoint mechanism inhibits CD8 T cell function in tumor microenvironment (223). Although immune checkpoint blockade molecules, anti-

CTLA-4 antibody and anti-PD-1 antibody, have not been proven currently to have the effectiveness, there is too little information to decide efficacy of ipilimumab and nivolumab in sarcomas. Thus, immune checkpoint blockade medicines should be evaluated in the future. Adoptive cell transfer approaches are also the subject of new sarcoma treatment trials. Overall, these trials and successes suggest that immunotherapy is moving to the forefront of therapy for bone and soft tissue sarcomas. All immunotherapies are reviewed in figure 3.1.

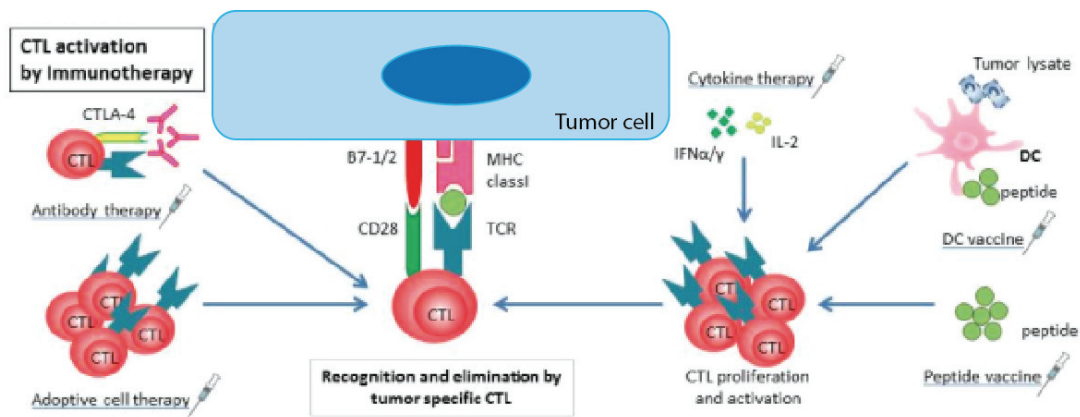


Figure 3.1: Immunotherapy against tumor cells. A number of strategies exist for induction of antitumor immunity against hepatocellular carcinoma cells. Antibody therapy using CTLA-4, adoptive cell of CTL specific to the tumor, cytokine therapy using INF or IL-2 to stimulate a CTL response and peptide/DC vaccine. Tumor-specific cytotoxic T lymphocytes (CTLs) activated by various immunotherapies are capable of recognizing and eliminating cancer cells. (224)

4. Aim of the thesis

As it was highlighted in the bibliographic section of this manuscript, chondrosarcoma is a complex malignancy with a spectrum of histological differentiation and a spectrum of malignant potential. Because of their extracellular matrix, low percentage of dividing cells, and poor vascularity, chondrosarcoma are relatively chemo- and radiotherapy resistant. To date, adequate surgical excision remains the most important principle of treatment for patients with chondrosarcoma. Improving chondrosarcoma clinical management is therefore a challenging problem and novel therapeutic approaches are needed to decrease tumor relapse and increase patient's survival and quality of life.

Recent scientific data in the microenvironment and molecular pathways involved in chondrosarcoma have highlighted the role of matrix metalloproteinases, bone niche, hypoxia-inducible factor (HIF), and mTOR (32-34). Treatments targeting tumoral microenvironment or signalling pathway have therefore been suggested as complements to surgical excision (31, 35, 225). Some of these approaches are currently under investigation in clinical trials.

Immunotherapeutic approaches are other options that could be considered for chondrosarcoma. Immunotherapy has regained interest over the last few years and cancer immunotherapy was Science magazine's 2013 advance of the year {Couzin-Frankel, 2013 #4596}. Passive immunotherapy (i.e., monoclonal antibodies) has proven remarkably successful in selected cancer subtypes, such as lymphoma. Strategies to reactivate antitumor immune responses with drugs targeting CTLA-4 and PD1/PD-L1 have led to striking advances in the therapy of melanoma, some carcinomas, and hematopoietic neoplasias. Nevertheless, immunotherapy strategies for people with sarcomas (recently reviewed (214)) are comparatively less advanced. Evidence from mouse models (226) and the observation that immunosuppressed patients triple their incidence of (non-Kaposi) sarcomas (227) support a major role for the immune system in preventing the development and progression of these cancers.

Osteosarcoma represents a specific sarcoma in which immunotherapy has proven effective, such that regulatory authorities have approved the non-specific

immune stimulator MTP (muramyl tripeptide) for human use in primary osteogenic sarcoma. New strategies based on clinically approved immune checkpoint inhibitors and specific targeting of tumor antigens using adoptive transfer and chimeric antigen receptor techniques have also been developed. Keeping in mind/in view the promises and hopes generated by immunotherapeutic approaches targeting immune checkpoints like PD-L1, it appears that there is an immediate opportunity to assess the potential of such strategies in the spectrum of human chondrosarcomas.

Chondrosarcoma immune microenvironment and the implication of immune checkpoint in this group of tumors remaining largely unknown, my PhD project aimed at investigating chondrosarcoma immunological microenvironment, its implication in chondrosarcoma growth and response to therapy. Moreover, we planned to further explore the role played by immune checkpoint PD-1/PD-L1 in chondrosarcoma and determine whether targeting PD-1/PD-L1 pathway targeting alone or in combination with conventional/targeted therapies could be an innovative immune-based therapeutic strategy for CHS patients.

The results of this work will be presented in the coming paragraphs and the results will be divided into five main sections.

1. Characterization of the immunological niche on human tumor biopsies at the cellular level (T lymphocytes, macrophages, PD-1) and the expression of PD-L1 on human chondrosarcoma cells
2. Characterization of the immunological niche on Swarm rat chondrosarcoma model (SRC) and comparison to the human chondrosarcoma immune environment.
3. Analysis of main immune populations (T lymphocytes and macrophages) involvement in chondrosarcoma progression. Given the difficulty to gain access to sufficient human CHS biological material, this study will be conducted in the SRC model.
4. Effect of PI3K/Akt/mTOR pathway inhibition on chondrosarcoma immune response. Here we will investigate whether drugs targeting the

PI3K/Akt/mTOR pathway-targeting agent could exert an immunostimulatory effect and initiate an immune response in chondrosarcoma. For this purpose, we will look at the changes in immune cells populations and cytokines secretion arising in response to the evaluated treatments.

5. Development of preclinical studies to determine the role of the PD-1/PD-L1 pathway in relevant syngeneic CHS animal model, and determine whether a strategy using blocking mAb anti-PD-L1 is applicable in SRC.

This study will allow the characterization to better understanding of the chondrosarcoma immunological niche and determine its potential role in tumor progression and response to targeted therapy. Understanding this relation between the chondrosarcoma and its immune environment will help us to better characterize and treat this complex tumor. This project would open the way to novels approaches of combined therapies associating immunotherapy and targeted therapy for the treatment of resistant or refractory bone sarcoma

5. Materials and methods

5.1 Cell cultures

The human chondrosarcoma (SW1353), rhabdomyosarcoma (SJCRH30), bone marrow (K-562), Natural Killer (NK-92) cell lines, and rat osteosarcoma cell line (UMR-106) were all obtained from the ATCC (LGC Standards, Molsheim, France) and were cultured at 37°C, under 5% CO₂, using DMEM or RPMI medium (for NK-92 cell line and SJCRH30) 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 chondrosarcoma cells and chondrocytes were isolated from SRC or healthy animals by collagenase digestion (228) and cultured in DMEM medium supplemented with 10% FBS, 2% L-glutamine, and penicillin/streptomycin solution (10,000 U/ml; 10,000 µg/ml).

5.2 The induction of PD-L1 *in vitro*

The induction of PD-L1 expression in cultured cell lines was performed using 20 ng/ml INF γ (Sigma Aldrich, St-Louis, USA) in culture medium over night. Cells were incubated with PI3K inhibitor GDC-0941 (100 nM, Genentech, San Fransisco, Ca, USA), Akt inhibitor G-594 (100 nM, Genentech), mTOR inhibitor Everolimus (10 nM, Novartis, Bâle, Switzerland) or DMSO over night. The next day, cells were used to performed immunoblotting or FACs analysis.

5.3 *In vivo* assay

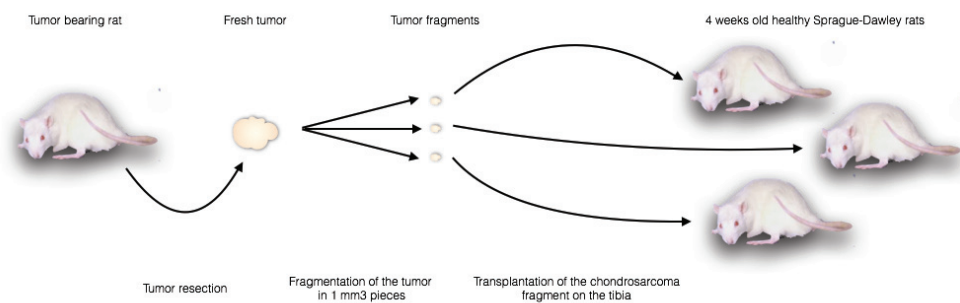
5.3.1 *Animal models*

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 Swarm rat chondrosarcoma model (SRC) has been previously described (38) (Fig 5.1). This model is a grade II chondrosarcoma with mild cellular atypia. It mimics its human counterpart in terms of aggressivity and chemo resistance. Tumors were grafted on 25-days-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 periosteal abrasion. Upon establishment of palpable tumors, i.e. 10 days after tumor implantation, animals were randomly divided into treatments groups. Two kinds of treatments were performed and will be detailed in the following paragraphs:

Implantation procedure



SRC model timelapse

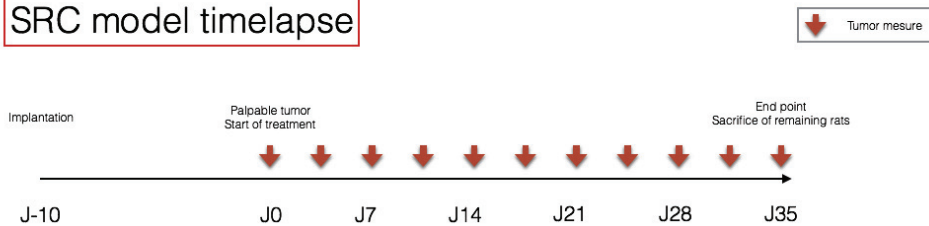


Figure 5.1: Transplantable orthotopic Swarm rat chondrosarcoma. The figure represent the implantation procedure of SRC and the time lapse of the of the model

5.3.2 Evaluate the effects of PI3K, Akt and mTOR inhibitors on immune infiltrates and immune response

PBS (100 ul; n=17), PI3K inhibitor Taselisib (200 mg/kg, Genentech; n=17), Akt inhibitor Ipatasertib (100 mg/kg, Genentech; n=17), mTOR inhibitor Certican (1 mg/kg, Novartis; n=17). Treatments were administered orally twice a week.

5.3.3 Selective depletion of immune populations were performed using chemical agents

PBS (1 ml; n=14), Azathioprine (2mg/kg; n=14; Sigma Aldrich, St-Louis, USA), Liposomal Mifamurtide (0,1 mg/kg; n=14; Adooq bioscience, Irvine, USA), Liposomal Clodronate (50 mg/kg; n=14; Clodronateliposomes.com, Amsterdam, The Netherland). Treatments were administered IP 5 days a week for azathioprine, twice a week for the other groups. The effect of the 3 immunomodulator treatments are resume in figure 5.2.




Model	Treatments	Targets	Effects
	Clodronate liposome		Toxic action on phagocytic cells Macrophages and monocytes
	Mifamurtide Liposome	Macrophages	Pro-inflammatory action on phagocytic cells. M2 to M1 conversion
	azathioprine		Lymphocytes

Figure 5.2: The targets and effects of each immunomodulator treatment.

For each protocol, the animals were treated over a period of 5 weeks or till tumors reached 2000 mm³. Tumor growth was monitored by regular visual

inspection and tumor dimensions were measured every 2-3 days. Tumor volume was calculated using the formula:

Volume = longest tumor diameter x (shortest tumor diameter)²/2.

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:

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

Kaplan–Meier analysis was carried out 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.

At the completion of treatment for all experimentations, rats were sacrificed, tumors and spleens harvested. Part of tumors and spleen were used for chondrosarcoma cells isolation and splenocytes harvesting while 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.

5.4 Immunoblotting

Immunoblotting was performed to evaluate PD-L1 expression in bone sarcoma cell lines. Proteins extraction and immunoblotting procedures have been previously reported (77). All proteins were detected, after dosing, by resolving proteins on Criterion Mini Protean 4–15% SDS-PAGE (Bio-Rad Laboratories, Marne La Coquette, France) and blotted onto nitrocellulose membrane. Primary antibodies against PD-L1 (AHP1703, AbDSeroTech, 1:2000) and β -actin (Cell signaling, 1:3000) were used. Immunoreactive bands were visualized by using ECL Plus (GE Healthcare, Orsay, France) and Bio-Rad ChemiDoc MP system (Bio Rad) after incubation with the horseradish peroxidase–conjugated anti-

rabbit (1:10000) for PD-L1 or anti-mouse (1:3000) for secondary antibodies (Cell signaling, Danvers, MA, USA). Quantitative analyses of the signals were performed with Image Lab (Bio Rad).

5.5 Splenocytes harvesting

Spleens were mashed on 40- μ m-cells strainer (BD Bioscience, San Jose, USA) placed on top of a 50 mL Falcon tube, using a syringe plunger. The collected cells were centrifuged (250 g, 5 min, 4°C) after addition of 10 mL RPMI medium supplemented by 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 (250 g, 5 min) and resuspended in complete RPMI medium. Splenocytes were either used immediately for cytotoxicity assays or frozen in RPMI medium supplemented with FBS (50%) and DMSO (10%) and stored in liquid nitrogen for further analyses.

5.6 Cytotoxicity assay

A nonradioactive, fluorometric cytotoxicity assay using calcein–acetoxymethyl (calcein AM; Thermofisher C3099) was performed to measure the lysis of rat chondrosarcoma cells and chondrocytes (further named target cells) by splenocytes. Target cells (1×10^6 /ml) were labeled with Calcein-AM (10 μ mol/L) at 37°C for 1 h before being washed twice in complete culture medium and plated at 10^4 cells/wells in round bottom 96-well microtiter plates (Nunc, P8116, Sigma Aldrich, St-Louis, USA). Target cells were then incubated with 400 μ mol/L Sulfinpyrazone (Sigma-Aldrich) in complete medium (1h, 37°C) to avoid spontaneous calcein release.

Splenocytes isolated from animals of all treatment group 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 6h 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 6h under normal culture conditions.

Supernatants were then collected (100 µl) by centrifugation (250 g; 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 (229). For each sample, calcein release was measured on Tecan Infinite F500 (Tecan group ltd. Switzerland) (excitation filter: 485 ± 9 nm; band-pass filter: 530 ± 9 nm). Specific lysis induced by the effectors was calculated according to the formula:

Specific sample lysis= $[(\text{sample release} - \text{spontaneous release})/(\text{maximal release} - \text{spontaneous release})] \times 100$. All tests were run in triplicate.

5.7 Measurement of cytokines secretion

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

5.8 Phenotypic analyses of chondrosarcoma immune microenvironment

Chondrosarcoma immune microenvironment was analyzed from 52 SRC and 26 conventional grade 1 to grade 3 human chondrosarcoma samples treated in our institution between 2004 and 2012. All samples were fixed in 4% formalin (pH 7.4) and embedded in paraffin. Deparaffination 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), PD-1 (1/100), PD-L1 (1/300) (respectively OX-8; ED2; AHP1706; AHP1703; AbD SeroTech, Oxford, UK). CD8 and CD163 primary antibodies were revealed using an unconjugated goat anti-mouse secondary antibody and CD3, PD-1, PD-L1 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 chondrosarcoma samples, 5 µm sections from FFPE blocks were deparaffinized and rehydrated. Immunostaining for CD3, CD8, CD4 and CD163 was performed on 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) (PD-1 and PD-L1 antibodies (AHP1706 and AHP1703 from Abd Serotec) were applied at 1/100. 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).

All slides were analyzed using a Nikon microscope (EclipseNi-E Nikon Corp)

5.9 Quantitative analysis of chondrosarcoma immune infiltrates

Quantitative analysis of chondrosarcoma immune infiltrates was similarly conducted on SRC and human chondrosarcoma slides. Chondrosarcoma'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 to 25% of positive cells were encountered in the field; 2 when 26 to 50 % positive in the field; 3 when we encounter 50% and more positive cells.

5.10 *In vitro* blocking of PD-L1

To investigate the role of PD-1/PD-L1 checkpoint in chondrosarcoma, it is necessary to block the interaction of PD-L1 with its receptor PD-1 *in vivo* and study the consequences of this blocking on tumor progression and immune environment. Nevertheless, there is a huge pitfall. No PD-L1 blocking antibody crossreacting with the rat species is yet available. Therefore we examine the possibility to block rats PD-L1 using a mouse PD-L1 antibody.

UMR-106 rat osteosarcoma cell line was used for the blocking of PD-L1 *in vitro*. Cells were treated or not with 20 ng/ml of INF γ over night for the upregulation of PD-L1. We then add 10 μ g of PD-L1 *in vivo* ready blocking antibody (10 μ g BioXcell, West Lebanon, NH, USA) or rat *in vivo* ready IgG2 isotype control (10 μ g LTF-2, BioXcell) was added to the cell culture for 1h on ice. After being washed with PBS, cells were marked with a mouse PD-1Fc chimera (10 μ g RnDsystems, Minneapolis, MN, USA) chimera or isotype control human IgG1 isotype control (10 μ g, RnDsystems), both coupled to CF488a (Mix-n-stain antibody labeling kits, Biotium, Hayward, Ca,USA). The analyses were performed on 10 000 events on a 4 color Facscalibur cell analyzer (BD Biosciences, San Jose, Ca, USA) and cellquest program (BD Biosciences).

5.11 Statistical analysis

All the data are reported as the sample mean \pm the standard deviation (SD). Pairwise comparisons between means of different groups were performed using a Student 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 is considered statistically

significant if the Student t-test gives a significance level (P value <0.05 . Multiple comparisons were performed using a univariate analysis of variance (ANOVA).

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

6. Results

6.1 Human chondrosarcoma immune microenvironment

6.1.1 Description

The localization, lineage and density of immune cells present in chondrosarcoma microenvironment were analyzed in a cohort of 26 grade 1 to 3 conventional chondrosarcoma.

On each tumor, positive cells for each immune marker considered (CD4, CD8, CD163) were counted on 5 different areas located at the margin of cartilaginous nodules and surrounding muscles.

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 6.1).

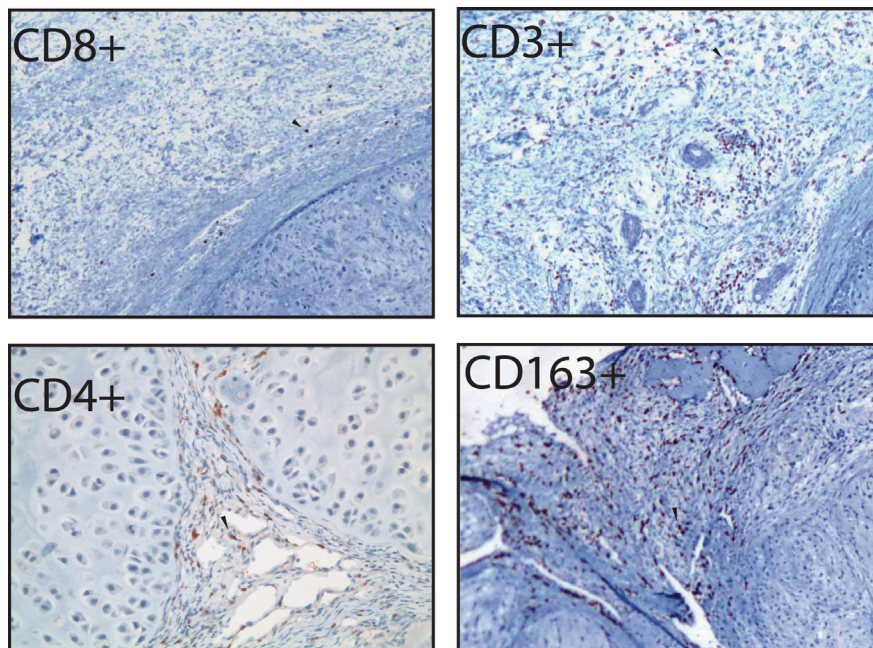


Figure 6.1: Immune infiltrate in human chondrosarcoma. IHC staining for CD8, CD3, CD4, CD163 indicated the presence of CD8+, CD3+, CD4+, CD163+ cells (black arrow) at the periphery of tumors in human chondrosarcoma (original magnification 200X).

The density of lymphocytes and macrophages varied among patients and ranged from minor to dense (Table 6.1). The highest density of T cells was observed in grade II chondrosarcoma. The density of CD163+ cells ranged from negligible to important (Table 6.1); the highest density of CD163+ cells was found in grade III chondrosarcoma (Table 6.1).

Table 6.1: Clinical information of chondrosarcoma patients

Patient info										Average infiltrates cells / tumors cells on 400X area				
Severity score	Patient file #	Sex	Initial size (mm)	Grade	Age	Quality of surgery	Relapse time (months)	Time before death (months)	PDL1 expression	Average CD163	Average CD8	Average CD4	Average CD3	CD8/CD3 ratio
+	1102924	F	80	1	50	R0	NA	NA	++	22,32	29,52	34,92	54,12	1,32
+	611387	M	50	1	62	R1	32	NA	++	27,72	31,2	30,24	60,96	1,12
+	500904	F	Unknown tumor size	1	76	R1	NA	NA	+	11,52	28,8	24	52,44	2,5
+	911280	M	100	1	76	R0	NA	NA	+++	34,32	51,96	63,96	99,24	1,51
+	8404572	M	70	1	78	R0	NA	NA	++	28,08	30,9	33	68,55	1,10
++	905554	M	45	2	56	R0	47	NA	++	29,52	59,76	585,84	88,8	2,02
++	1002924	M	80	2	45	R0	NA	NA	+	35,04	60,48	28,08	81,24	1,72
++	1011564	M	80	2	53	R0	NA	NA	+	19,08	64,08	22,44	88,32	3,35
++	608940	M	100	2	55	R0	NA	NA	++	26,88	44,76	30,12	87,84	1,66
++	811636	M	110	2	69	R0	59	NA	+	22,8	49,08	23,4	74,4	2,15
++	1001193	F	33	2	25	R0	9	NA	+	27,12	64,8	21,48	82,56	2,38
++	800144	F	40	2	49	R1	NA	NA	+++	33,24	63,84	54,12	127,56	1,92
+++	1102370	M	70	2	68	R0	NA	NA	++	67,2	37,2	52,8	88,32	0,55
+++	704120	F	125	2	79	R0	NA	NA	+	52,44	23,52	11,52	38,16	0,44
+++	812859	F	120	2	47	R0	NA	NA	++	41,88	16,8	15,6	41,88	0,40
+++	906576	M	100	2	35	R1	NA	NA	++	48,84	11,28	23,04	39,24	0,23
+++	1105396	M	85	2	77	R1	NA	NA	+++	56,52	23,88	23,64	48	0,42
+++	803710	F	100	2	17	R0	NA	NA	++	328,32	43,32	86,28	126,48	0,13
+++	807793	F	70	2	34	R0	NA	NA	+	52,2	10,68	22,2	33	0,20
+++	602287	M	150	2	70	R1	8	24	++	60,84	14,16	22,92	38,88	0,23
++++	9204589	M	240	2 to 3	63	R0	11	99	++	82,92	10,8	10,56	24,48	0,13
++++	1205144	M	70	2 to 3	75	R0	NA	NA	+++	61,68	51,72	38,4	75,96	0,83
++++	1004095	F	70	3	82	R1	2	6	+	29,52	13,56	14,64	19,8	0,45
++++	405607	M	100	3	18	R1	3	9	++	74,76	8,16	10,92	21,24	0,10
++++	700281	M	150	2 to 3	67	R0	NA	NA	+++	59,52	49,68	15,36	75,6	0,83

6.1.2 Implication of immune infiltrate in chondrosarcoma evolution

A) Immune infiltrate and patients survival

Immune infiltrate density was correlated with tumor aggressivity and patients survival.

Indeed, patients with above the median 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 6.2) and tended to have better PFS ($P > 0,05$) (Fig 6.3).

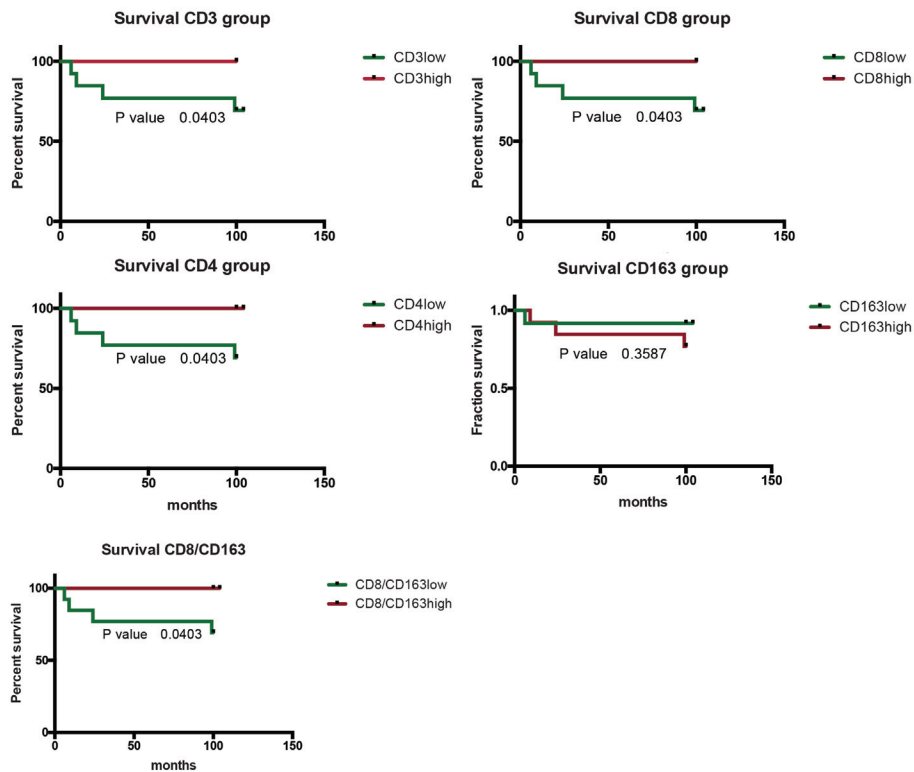


Figure 6.2: Immune infiltrates correlation with chondrosarcoma's survival. Each graph presents the correlation between one immune population and patients survival. Patients with above the median CD3+, CD4+ CD8+ cells have better survival than patients with below the median T cell counts. Interestingly CD163+ cells count did not correlate with patients survival.

Interestingly, CD163+ cells infiltrations were not correlated with patients overall survival nor with tumor relapse (Fig 6.2 and 6.3).

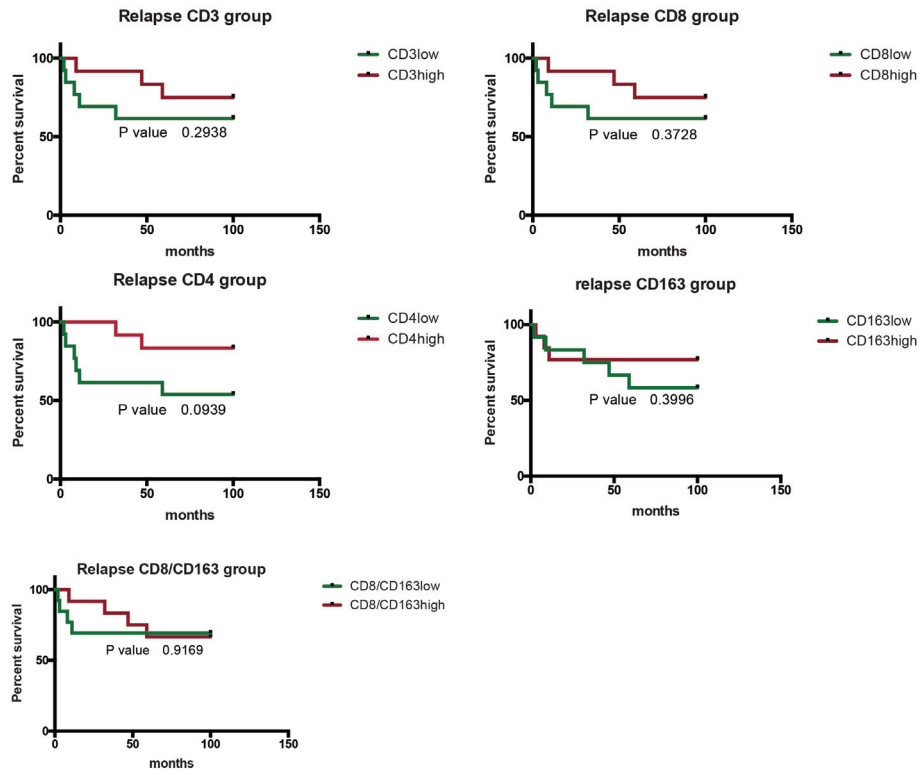


Figure 6.3: Immune infiltrates correlation with chondrosarcoma's progression. CHS relapse was evaluated based on immune cell counts. Patients with above the median CD3+, CD4+ CD8+ cells tended to relapse later than patients with below the median T cell counts; whereas CD163+ cells count did not correlate with tumor relapse.

B) Relation between immune infiltrate and tumor size

Interestingly, in this series of 26 conventional chondrosarcoma, an inverse correlation between high CD8 + T cells count and tumor size at the resection time was observed, whereas a correlation between high CD163+ cell count and tumor size was observed (Fig 6.4).

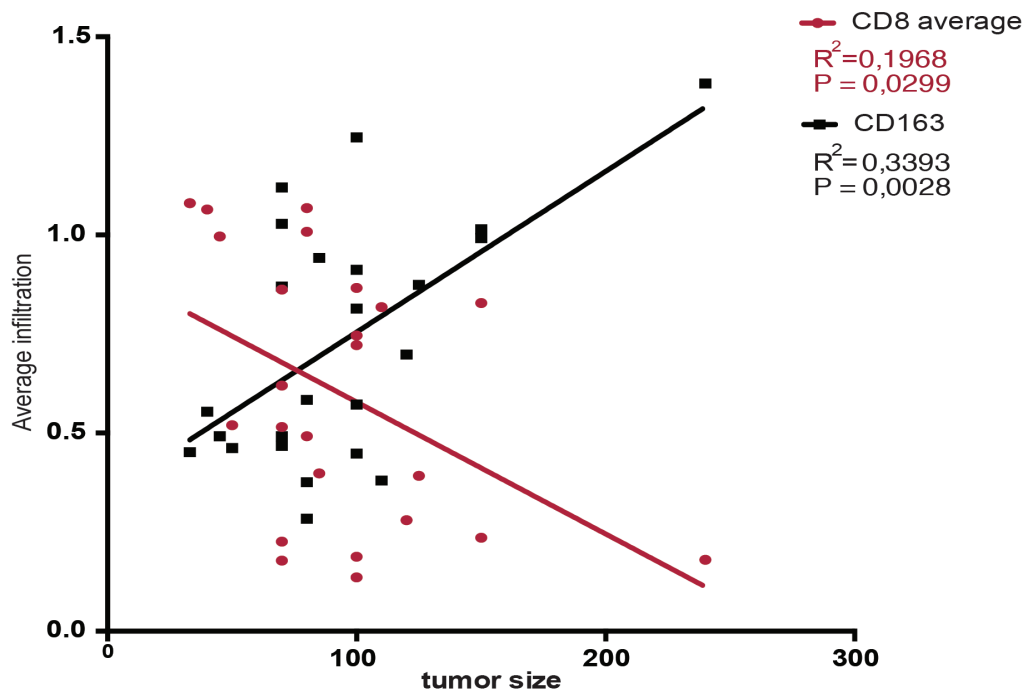


Figure 6.4: Correlation between CD8+, CD163+ cells counts and tumor size. In this series of 26 conventional chondrosarcoma, an inverse correlation between CD8+cells count (red dots) and tumor size (in mm³) was found; whereas a correlation between CD163+ cells (black dots) and tumor size was encountered.

C) Relation between immune infiltrate and tumor aggressivity

The correlation between tumor grade, invasiveness and immune cells counts was studied in this series of 26 conventional chondrosarcoma. The less aggressive tumors had the highest CD8+ cells count and lowest CD163+ cells counts while tumors of higher grades and infiltrative tumors had the lowest CD8+ cells and highest CD163+ cells counts (Fig 6.5).

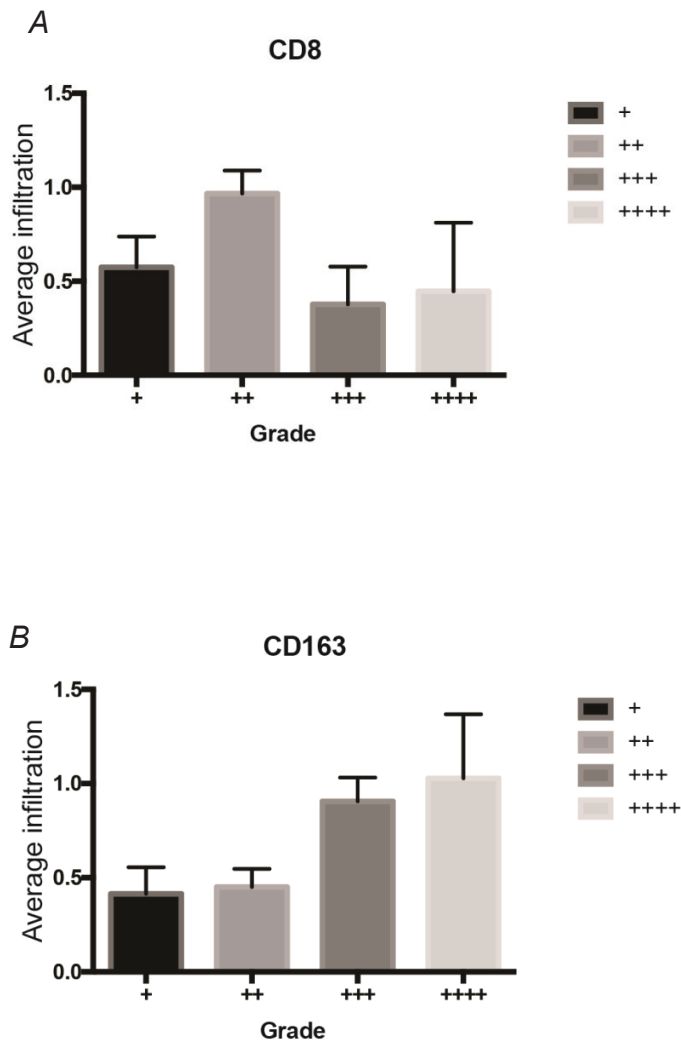


Figure 6.5: Correlation between CD8+, CD163+ cells counts and tumor aggressivity. The correlation between CD8+ cells infiltrate (A) or CD163+ cells infiltrate (B) and tumor aggressivity (expressed as tumor grade) was evaluated. Chondrosarcoma are classified as follow: + grade I chondrosarcoma; ++ non-invasive grade II chondrosarcoma; +++ invasive grade II chondrosarcoma; ++++grade III chondrosarcoma

D) Inverse effects/implication of CD8+ T cells population versus CD163+ cells

For each patient, we evaluated, for each patient, the density of CD8+ cells infiltrates (effectors of immunity) versus the one of CD163+ cells to analyze if both populations could influence each other.

In patients showing an important CD8+ infiltrate, a low density of CD163+ cells was encountered and vice versa. This indicates that an inverse correlation between CD8+ and CD163+ cells count exists ($R^2=0.2$, $p=0.035$) (Fig 6.6).

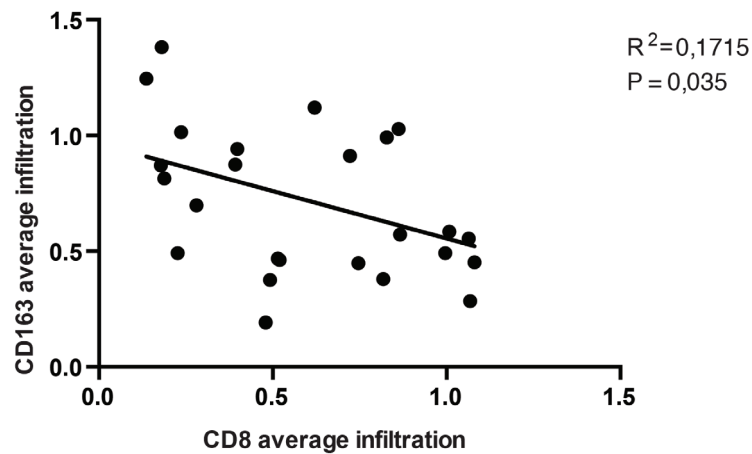
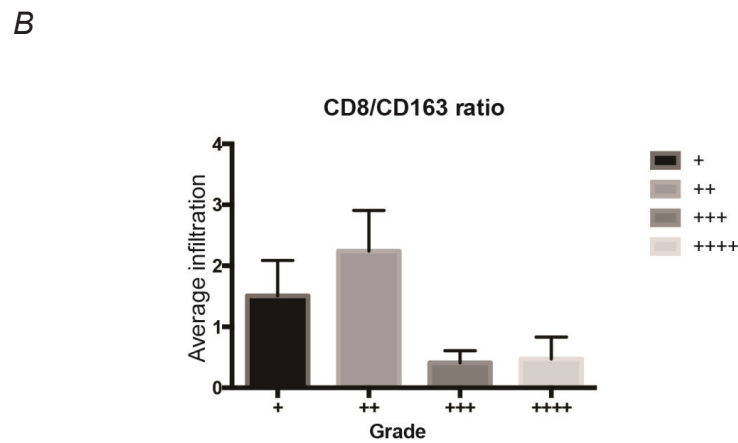
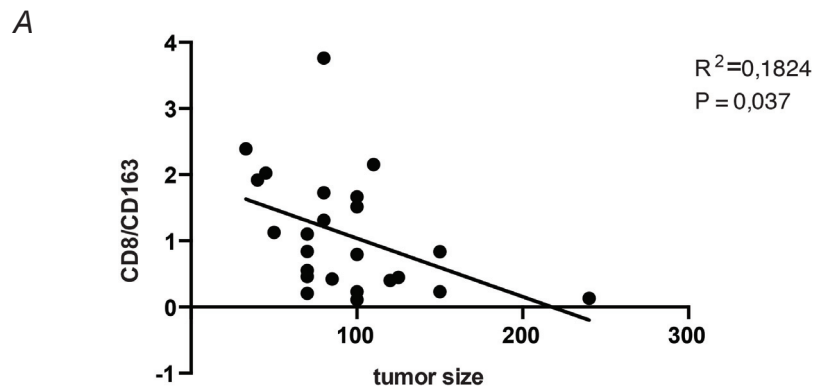


Figure 6.6: Correlation between CD8+ lymphocytes and the CD163+ macrophages in tumor's microenvironment. Each dot represents one of the 26 conventional chondrosarcoma of this study.

The CD8/CD163 ratio, i.e. the number of CD8+ T cells over M2 related macrophages count (30) was calculated and found to be inversely correlated with tumor size (Fig 6.7A) and tumor grade (Fig 6.7B and 6.7C). The less aggressive tumors had the highest CD8/CD163 ratio while Grade 3 and Grade 2 infiltrating tumors had the lowest CD8/CD163 ratio.



C

Tumor aggressiveness	Histological grade	Characteristic
+	I	Few cells with no variation in size and shape, without signs of peripheral tissues invasion.
++	II	Hypercellularity. The cells show variation in size and shape with extensive myxoid matrix component, without signs of peripheral tissues invasion.
+++	II	Hypercellularity. The cells show variation in size and shape with extensive myxoid matrix component, with signs of peripheral tissues invasion.
++++	III	High cellularity, with prominent pleomorphic appearance and atypia and signs of peripheral tissues invasion.

Figure 6.7: CD8/CD163 ratio and tumor aggressivity. Correlation between CD8/CD163 ratio tumor size at the time of resection (A) and tumor grade (B) was evaluated. A; Each dot represents one of the 26 conventional chondrosarcoma of this study. B: Chondrosarcoma are classified as follow: + grade I chondrosarcoma; ++ non-invasive grade II chondrosarcoma; +++ invasive grade II chondrosarcoma; ++++grade III chondrosarcoma

6.1.3 Take home message

- Lymphocytes and macrophages are found in the marginal area of human conventional chondrosarcoma.
- Lymphocyte infiltration is of good prognostic while macrophages is of bad prognostic for human conventional chondrosarcoma
- CD8 infiltration is inversely correlated to the CD163 infiltration.

6.2 Swarm rat chondrosarcoma immune microenvironment reflects human chondrosarcoma's

The analyses of chondrosarcoma immunological environment realized on 26 conventional chondrosarcoma samples indicate that the immune populations could be involved in chondrosarcoma progression. The implication of each immune population in chondrosarcoma progression cannot be conducted on human chondrosarcoma samples, partly because of the rarity of this tumor. Thus to conduct such analyses, an animal model recapitulating the human pathology and its immunological environment is necessary.

The Swarm rat chondrosarcoma model (SRC) mimics human chondrosarcoma in term of evolution, thus we characterized its immunological environment and conducted further analyses in this model.

6.2.1 Description

As in human chondrosarcoma, tumor immune infiltrate was analyzed on a “cohort” of 17 progressive SRC. On each tumor, positive cells for each immune marker considered (CD4, CD8, CD163) were counted on 5 different areas located at the margin of cartilaginous nodules and surrounding muscles.

CD4+, CD8+ T cells and CD163+ cells (monocytes/macrophages) were observed in all the 17 tumors and were mostly located in the peripheral area of the tumor (Fig 6.8).

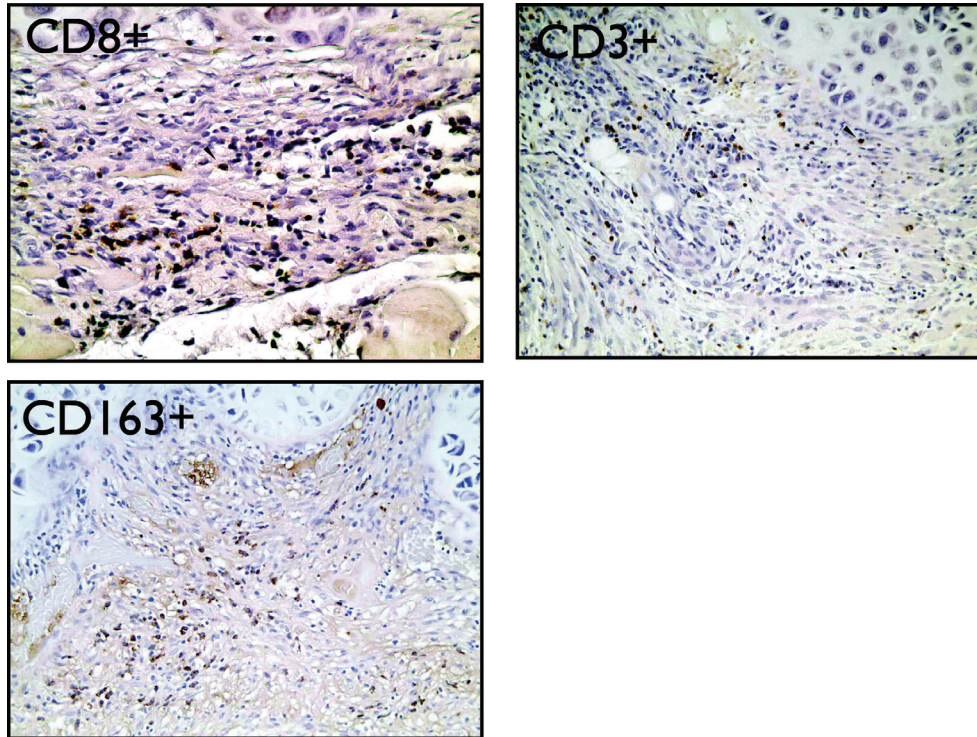


Figure 6.8: Immune infiltrate in SRC model. IHC staining for CD8, CD3, CD4, CD163 indicated the presence of CD8+, CD3+, CD4+, CD163+ cells (black arrow) at the periphery of tumors in rat chondrosarcoma (original magnification 200X).

6.2.2 Correlation between immune infiltrates and tumor progression in SRC

As in human chondrosarcoma, CD8+ cells count was found to be inversely correlated with tumor size (measured at the time of animal's sacrifice) whereas CD163+ cells count was correlated with it (Figure 6.9). In the SRC model the CD8/CD163 ratio was inversely correlated with tumor size (Figure 6.10).

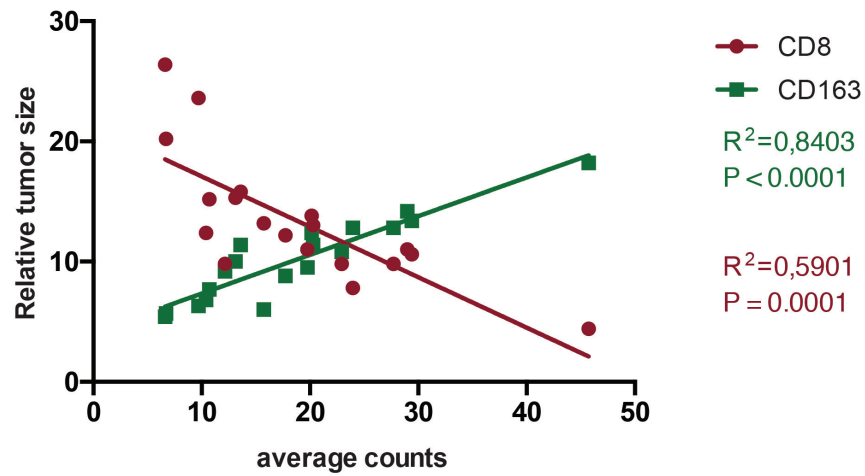


Figure 6.9: Correlation between CD8+, CD163+ cells counts and tumor size. In this series of 17 progressive, an inverse correlation between CD8+cells count (red dots) and relative tumor size (Size at day 21/size at day 0) was found; whereas a correlation between CD163+ cells (green dots) and tumor size was encountered.

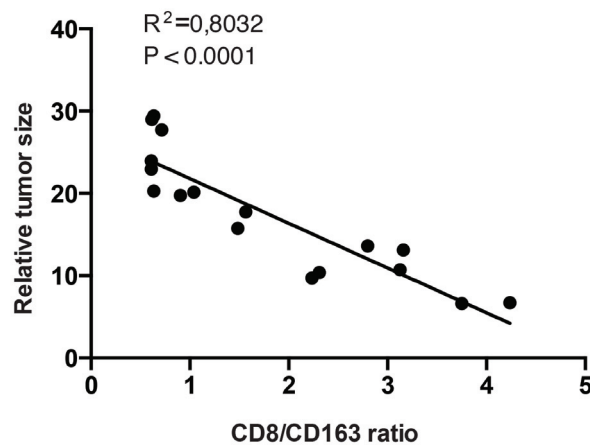


Figure 6.10: CD8/CD163 ratio and tumor agressivity. Correlation between CD8/CD163 ratio and tumor size at the time of sacrifice. Each dot represents one of the 17 progressive SRC of this study

6.2.3 Implication of T lymphocytes and macrophages in chondrosarcoma progression

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

T cell depletion was obtained using azathioprine an agent known to block the proliferation of lymphocytes and prevent the differentiation of naïve and memory T cells in effectors T cells (230). The macrophagic population was modulated in two ways:

- Clodronate was used to deplete the macrophage population (231).
- Mifamurtide, an agent currently used in bone sarcoma clinical trial, was used to force the conversion of pro-tumoral M2 macrophage to pro-inflammatory M1 macrophage (203).

The effects of each treatment on tumor progression and immune environment were analyzed and are reported in the following paragraphs.

6.2.4 Effect of selective depletions on immune environment

All treatments affected SRC immune environment.

In animals treated by clodronate or mifamurtide, a lower infiltration of CD163+ cells in tumor microenvironment was observed. In azathioprine treated animals, CD8+ and CD3 T cells were significantly decreased in tumor's environment (Fig 6.11).

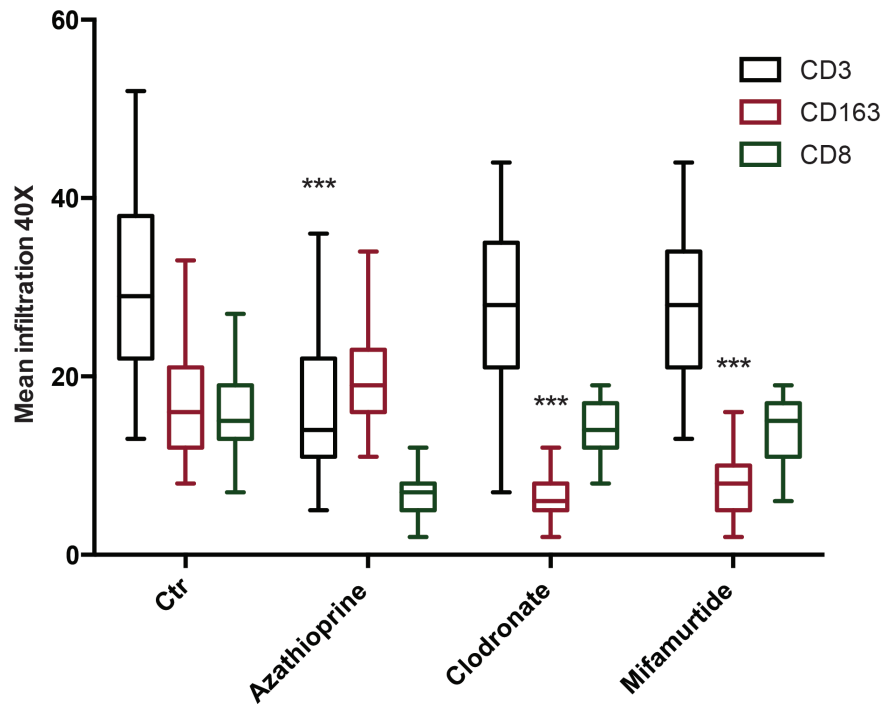


Figure 6.11: Effects of selective depletion on SRC immune infiltrates. Average immune cells infiltration (CD3+, CD8+, CD163+) in each treatment groups at the end of the experimentation. Azathioprine caused a decrease in CD3 and CD8+ cells whereas mifamurtide and clodronate affected CD163+ cells. Counts were made on 5 areas / tumor (400X magnification).

6.2.5 Effect of selective depletions on tumor progression

A) Macrophage population modulation

Both clodronate and mifamurtide affected tumor progression. Animals treated with these agents had significantly smaller tumors compared to control animals (respectively $1127 \pm 177 \text{ mm}^3$ and $1294 \pm 195 \text{ mm}^3$ versus $1913 \pm 202 \text{ mm}^3$ $P < 0,05$ on day 18). In rats treated with clodronate or mifamurtide, the tumor growth rate was lower than in the control group: tumor proliferation rate was respectively $16,23 (\pm 2,19)$ in the clodronate treated group and $24,32 (\pm 3,42)$ in the mifamurtide treated rats versus $63,17 (\pm 14,32)$ in the control group at day 18 ($P < 0,005$) (Fig 6.12A).

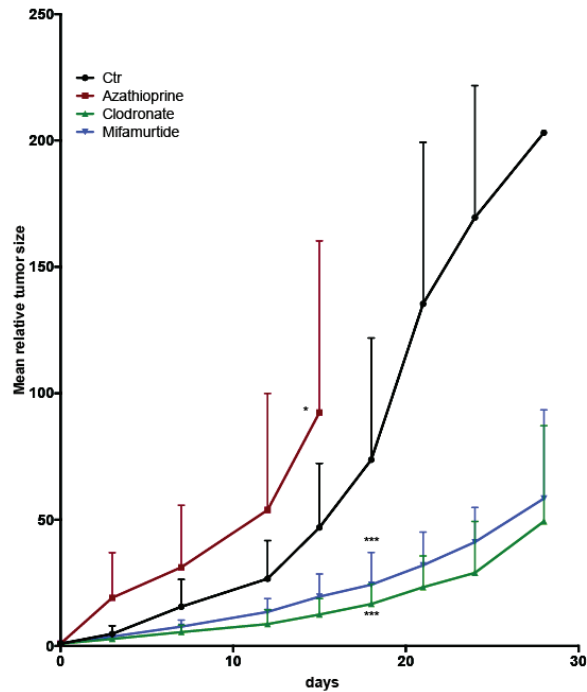
Both agents significantly delayed tumor progression and time necessary to reach the maximum tumor volume (2000 mm³) (respectively 26,38 ±1,65 days and 22,71 ± 1,98 days versus 16,92 ± 0,70 days in the control group, p < 0,05) (Fig 6.12B). At day 34, when the assay was stopped, 3 animals remained in both of the “macrophage modulation” groups, whereas all the control animals had died.

B) Depletion of T lymphocytes

T lymphocytes depletion by azathioprine caused an increase in tumor progression. 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 6.12A). Animals from the azathioprine-treated group had a significantly higher tumor volume than the control groups at day 14 (respectively 2349 ± 86 mm³ versus 1434 ± 169mm³ (P < 0,0005).

As a consequence of the increase in tumor progression, azathioprine-treated rats reached the endpoint (i.e. tumor volume of 2000 mm³) sooner than the control group (12,33 ± 0,41 days versus 16,92 ± 0,70 days p < 0,0005) (Fig 6.12B). Fourteen days after initiation of the treatments all animals from the azathioprine treated group were gone whereas ten animals remained in the control group (Fig 6.12B).

A



B

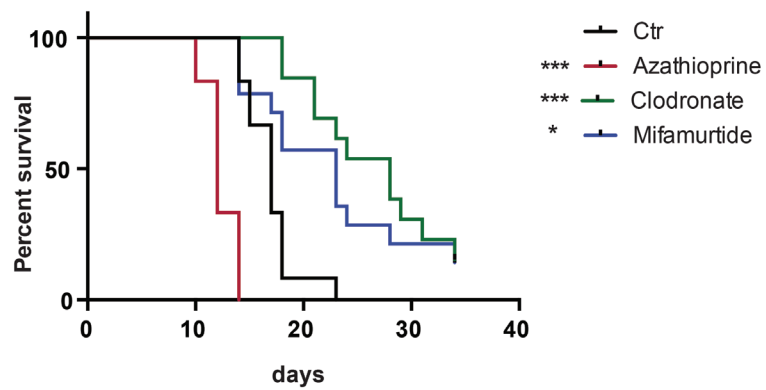


Figure 6.12: Effects of selective depletion on SRC progression. A: SRC evolution in control, CD3/CD8 depleted group (azathioprine treated), macrophages depleted (clodronate) or macrophages modulated (mifamurtide) groups expressed as relative tumor size. (*= $p < 0,05$; ***= $p < 0,005$). B: animal's survival in control and immune depleted groups (*= $p < 0,05$; ***= $p < 0,005$).

These data suggest that macrophages play a pro-tumoral action while lymphocytes repress chondrosarcoma growth possibly by specific cytotoxic activity directed against chondrosarcoma cells.

To gain more insight into the involvement of effector T cells and inflammation mechanisms in chondrosarcoma progression, the cytotoxicity of splenocytes from different treatment groups was analysed and production of pro-inflammatory cytokines was measured in rat sera.

6.2.6 Effectors T cells/cytotoxic activity of SRC splenocytes

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

Splenocytes isolated from healthy animals did not have a cytotoxic activity against chondrosarcoma cells (E:T ratio of 100:1, specific lysis of 2,5 % \pm 1,7) (Fig 6.13). Conversely, chondrosarcoma cells were lysed by splenocytes isolated from chondrosarcoma bearing rats: (27,0 \pm 6,5% was obtained at the E:T ratio 100:1). The splenocytes from azathioprine, treated rats had a lower cytotoxic activity on chondrosarcoma cells. (11,3 \pm 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 6.13).

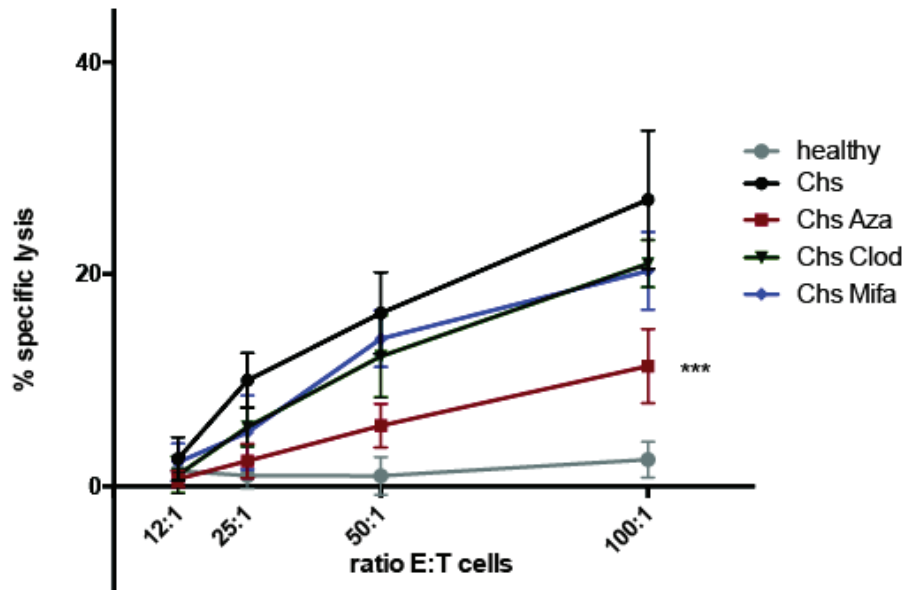


Figure 6.13: Cytotoxic response in chondrosarcoma. Splenocytes from healthy animals did not show a specific cytotoxic activity directed against chondrosarcoma cells. This activity was present only in animals with progressive tumor and was decreased by selective depletion of CD163 or CD3/CD8 cells (***) = $p < 0,0005$). Chs: progressive chondrosarcoma; Chs Aza: azathioprine treated SRC; Chs clod: clodronate treated SRC; Chs Mifa: mifamurtide treated SRC

These results confirm the hypothesis that effectors CD8+ T cells are located in chondrosarcoma microenvironment and play an antitumoral role and in a certain measure control chondrosarcoma growth.

6.2.7 Inflammation and chondrosarcoma progression

It is known that immunostimulatory and immunosuppressive cytokines that are involved in chondrosarcoma invasion and migration (30) are secreted in tumor microenvironment. Thus, we further ask whether chondrosarcoma could induce a systemic inflammation in the SRC model.

Four biological markers $INF\gamma$, $TNF-\alpha$, $IL-1\beta$ and $IL-10$, were analyzed on all animal sera.

IL-1 β and IL-10 were not detected either in healthy or chondrosarcoma bearing animals (Table 6.2).

	INF-y	TNF-a	IL-10	IL-1b
Healthy	< 32	< 47	< 10	<23
CTR	179,6 \pm 16,4	238,8 \pm 36,7	< 10	<23
Azathioprine	299,6 \pm 28,3	206,8 \pm 23,7	< 10	<23
Clodronate	318,5 \pm 41,4	159,3 \pm 18,4	< 10	<23
Mifamuridide	351,0 \pm 51,0	169,5 \pm 12,3	< 10	<23

Table 6.2: Inflammatory cytokines in chondrosarcoma. Serum levels of INF γ , TNF- α , IL10 and IL1 β in normal animals, progressive chondrosarcoma, CD163 or CD3/CD8 depleted chondrosarcoma-bearing rats. Data are given in pg/ml as the mean of each marker measured \pm SE, in each group N=7. Ctr: progressive chondrosarcoma; Azathioprine: azathioprine treated SRC; Clodronate: clodronate treated SRC; Mifamurtide: mifamurtide treated SRC

Two inflammatory cytokines INF γ and TNF- α were detected in the sera of all chondrosarcoma animals while undetectable in healthy animals (Table 6.2; Fig 6.14).

The three modulatory agents used (azathioprine, clodronate, mifamurtide) increased the level of seric INF γ compared to untreated SRC animals ($P < 0,05$) (Table 6.2; Fig 6.14A). None of the immunomodulator treatments caused changes in TNF- α level (Table 6.2; Fig 6.14B).

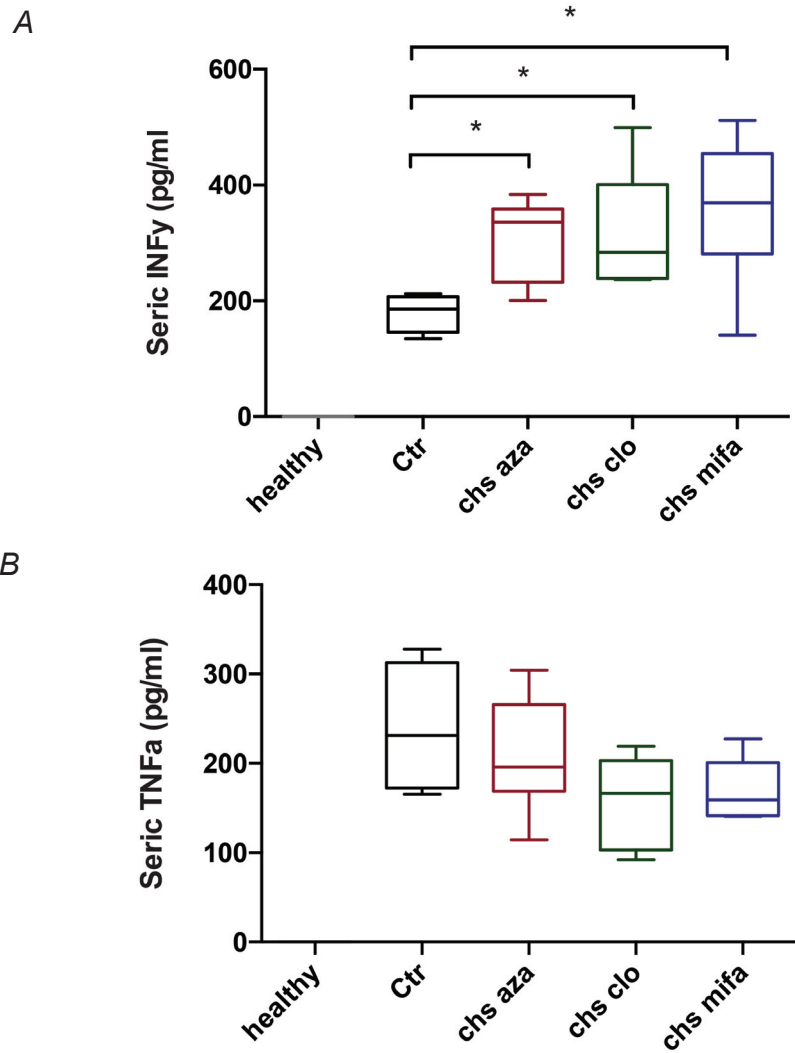


Figure 6.14: Inflammatory response in chondrosarcoma. Seric levels of INF γ (A) and TNF- α (B) in normal animals, progressive chondrosarcoma CD163 or CD3/CD8 depleted chondrosarcoma bearing rats (*= $p < 0,05$). Data are given as the mean of each marker measured \pm SE. Chs: progressive chondrosarcoma; Chs Aza: azathioprine treated SRC; Chs clod: clodronate treated SRC; Chs Mifa: mifamurtide treated SRC

6.2.8 Take home message

- Like in human, lymphocytes and macrophages infiltrate the marginal zone of SRC model.
- In SRC we showed by IHC and depletion that lymphocytes infiltrations are associated with a slower tumor growth.

- In SRC we showed by IHC and depletion/modulation that CD163 macrophages are associated with a faster tumor growth
- We show by cytotoxic assay and measure of cytokine that immunosurveillance is active in SRC.

The results in human and SRC model show that we could apply an immunotherapy to chondrosarcoma to boost the immune response toward the tumor. Before, we wanted to see if the inhibitors of the PI3k/mTOR pathway, that had given good results to slow down chondrosarcoma growth in the SRC model (77), could synergize with an immunotherapy.

6.3 Modulation of immune response in chondrosarcoma by PI3K pathway inhibitor

We evaluate the effect of agents targeting the PI3K/Akt/mTOR pathway on chondrosarcoma immune environment and assess whether a PI3K/mTOR pathway-induced immune response could contribute to the therapeutic efficiency of one of this pathway's inhibitors.

6.3.1 PI3K pathway inhibitors and tumor progression

The three inhibitors tested G-594: Akt inhibitor; GDC-0941: PI3K inhibitor; everolimus: mTOR inhibitor were used at concentrations previously determined as being the most effective.

There was evidence of antitumor efficacy for all the inhibitors tested; the animals receiving these treatments showed smaller tumors after 21 days of treatment in comparison to the control animals ($P < 0,05$) (Fig 6.15).

Among these agents, PI3K inhibitor (GDC-0941) appeared to be the most efficient agent as the tumor growth rate of this group was 13,56 ($\pm 3,39$) at day 21. The second most effective agent was the Akt inhibitor (G-594) which

efficiency on chondrosarcoma tended to be close to the one of mTOR inhibitor Everolimus (tumor growth rate respectively of 19,11 (\pm 3,76) and 25,71 (\pm 3,28)) at day 21. At this time point the tumor growth rate of the control group was of 40,88 (\pm 2,86) (Fig 6.15).

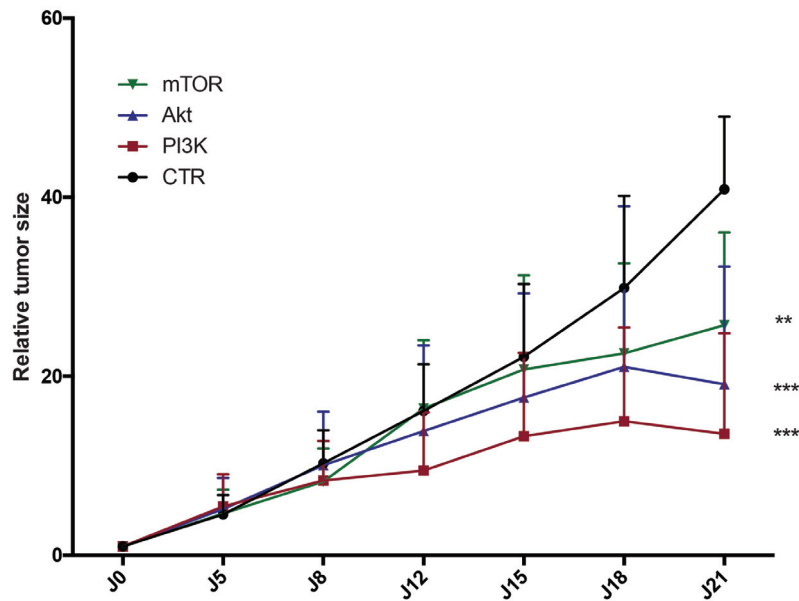


Figure 6.15: PI3K, Akt and mTOR inhibitors affect SRC progression. SRC evolution expressed as relative tumor size in control, GDC-0941 (PI3K inhibitor), G594 (Akt inhibitor) and Everolimus (mTOR inhibitor) treated SRC. (**: $p < 0,005$; ***: $p < 0,005$) (n=12). Ctr: progressive chondrosarcoma; PI3K: GDC-0941-treated SRC; Akt: G-594-treated SRC; mTOR: Everolimus-treated SRC

Similarly these agents increased animal's survival and time to reach the endpoint (2000 mm³). At the end of the experiment 33% animals remained in the control group, whereas respectively 73%, 75% and 70% remained in the GDC-0941, G-594 and Everolimus treated groups ($p < 0,05$) (Fig. 6.16).

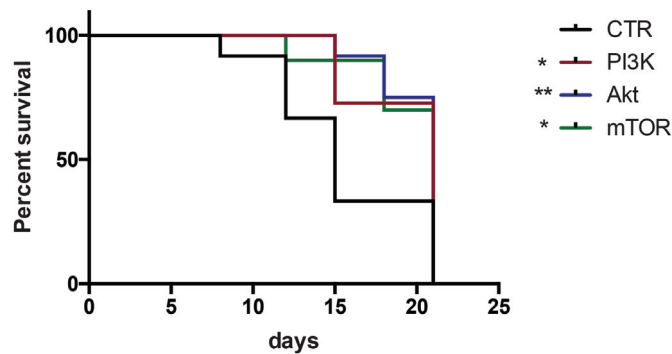


Figure 6.16: PI3K, Akt and mTOR inhibitors increase SRC survival. (*: $p < 0,05$; **: $p < 0,05$) (n=12). Ctr: progressive chondrosarcoma; PI3K: GDC-0941-treated SRC; Akt: G-594 treated SRC; mTOR: Everolimus-treated SRC

These results confirmed the possibility to use PI3K pathway inhibitors to slow down chondrosarcoma progression, as we previously shown for mTOR inhibitor (77).

To further investigate the effects of these inhibitors on chondrosarcoma immune microenvironment and the immune response, we analysed composition of SRC immune environment with a focus on CD163+, CD8+ and CD3+ cells.

The three PI3K pathway inhibitors evaluated did not affect SRC immune microenvironment composition. Similar densities of CD3+, CD8+ T cells and CD163+ cells (monocytes/macrophages) were observed in all tumors and were mostly located in the peripheral area of the tumor (Fig 6.17).

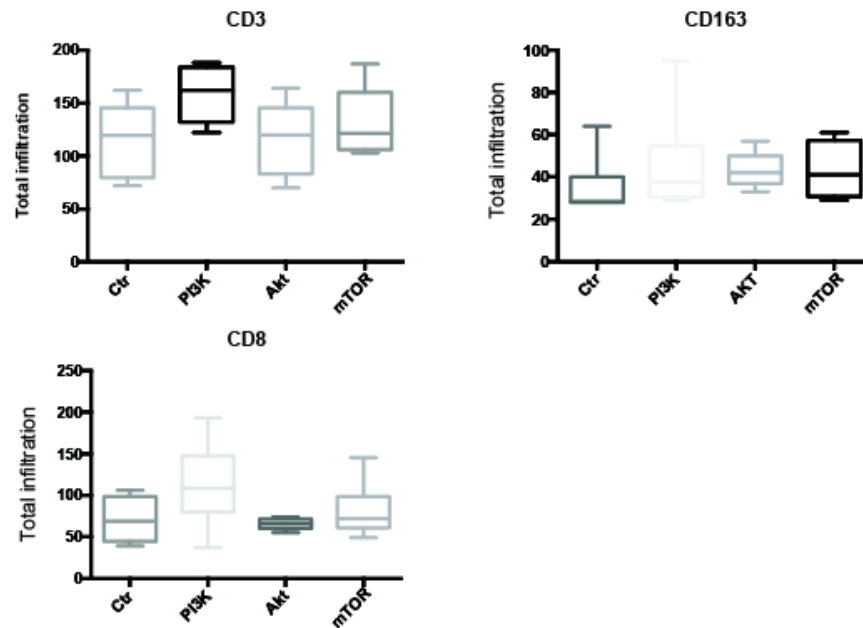


Figure 6.17: Effect of PI3K pathway inhibitors on SRC immune environment. Average Immune cells density for each treatment groups at the end of the experimentation. Immune infiltrate were counted in 5 areas (magnification x400). None of the inhibitor caused a change in immune infiltrate composition and density. Ctr: progressive chondrosarcoma; PI3K: GDC-0941-treated SRC; Akt: G-594-treated SRC; mTOR: Everolimus-treated SRC

We also studied the effect of these targeted therapies on cytotoxic immune populations and production of pro-inflammatory cytokines. These studies are presented in the following paragraphs.

6.3.2 PI3K pathway inhibitors and cytotoxic immune populations

The cytotoxicity of splenocytes isolated from healthy animals (n=6), progressive SRC (n=6), PI3K pathway inhibitors treated SRC (n=6 for each treatment) were evaluated against primary chondrosarcoma cells isolated from progressive untreated SRC.

As we found previously, splenocytes isolated from healthy animals did not have a cytotoxic activity against chondrosarcoma cells even at the E:T ratio of 100:1

(specific lysis of 1,3 % (\pm 0,3)) (Fig 6.18). On the opposite, chondrosarcoma cells were lysed by splenocytes isolated from animals bearing a progressive tumor: a significant lysis of 19,5 (\pm 0,8%) was obtained at the E:T ratio 100:1. The splenocytes isolated from all the PI3K pathway inhibitors treated groups showed a significant lesser cytotoxic action on chondrosarcoma cells than the one of the control group ($p < 0,005$). A specific lysis of respectively 10,84 % (\pm 1,3), 12,7% (\pm 0,9), 10,3 (\pm 1,5) was observed at the E/T ratio of 100 for the GDC-0941, G-594 and everolimus treated rats (Fig 6.18).

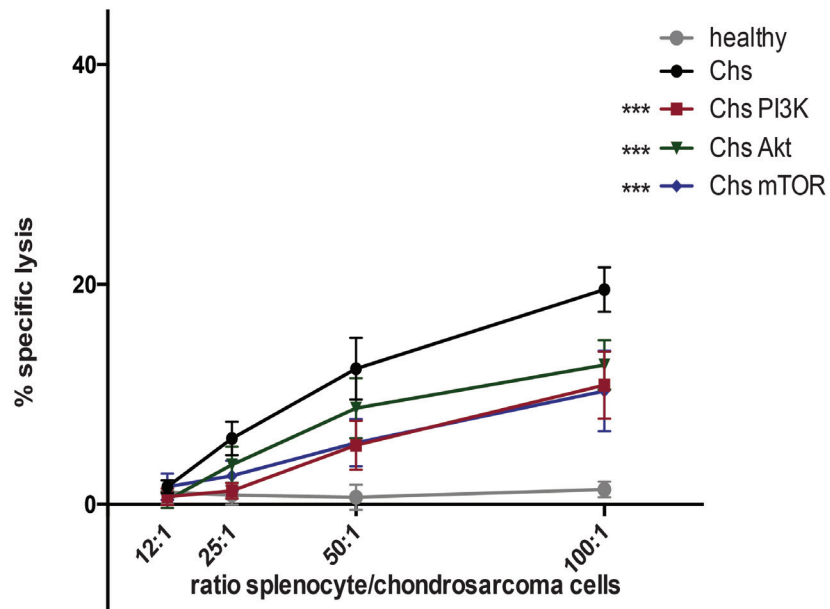


Figure 6.18: Effect of PI3K pathway inhibitors on cytotoxic response in chondrosarcoma. Splenocytes from healthy animals did not show a specific cytotoxic activity directed against chondrosarcoma cells. This activity was present only in animals with progressive tumor and was decreased by the PI3K pathway inhibitors used (***) ($p < 0,0005$).

Chs: progressive chondrosarcoma; Chs PI3K: GDC-0941-treated SRC; Chs Akt: G-594-treated SRC; Chs mTOR: Everolimus-treated SRC

6.3.3 PI3K pathway inhibitors and inflammation

The three PI3K pathway inhibitors used GDC-0941 (PI3K), G-594 (Akt), Everolimus (mTOR) all significantly decreased the level of seric IFN γ and TNF- α compared to untreated SRC animals (P < 0,05 for GDC-0941 and P < 0,005 for G-594 and Everolimus) (Fig 6.19). The fact that none of these cytokines were found in the sera of healthy animals indicate clearly that inflammation is caused by the tumor itself and that the variations observed happen in response to the corresponding treatments.

As we previously found, no IL-1 β and IL-10 were detectable in the sera of healthy animals, progressive tumor or either one of the PI3K pathway treated tumor (data not shown).

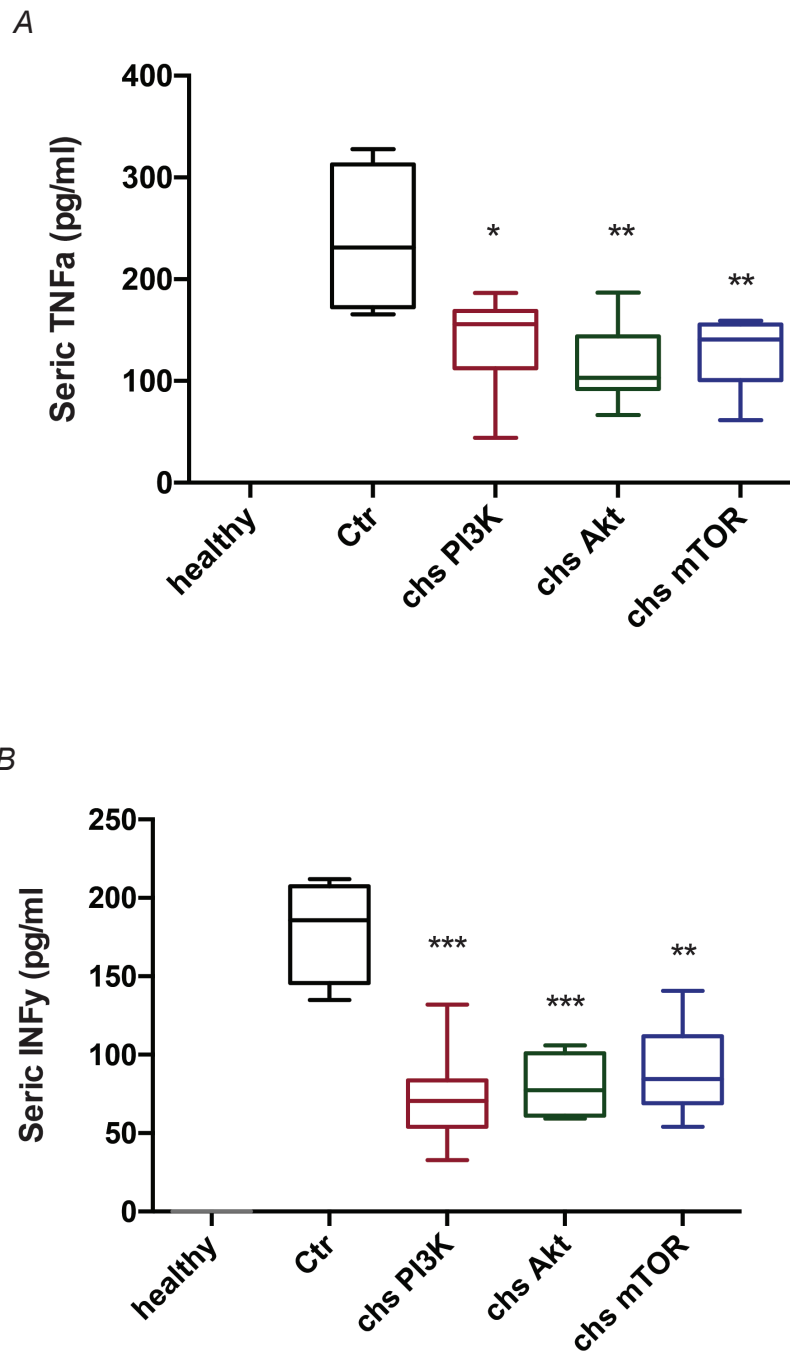


Figure 6.19: Effect of PI3K pathway inhibitors on inflammation in chondrosarcoma. Seric TNF- α (A) and IFN γ (B) levels measured in normal animals, progressive chondrosarcoma GDC-0941 (PI3K), G-594(Akt) and Everolimus (mTOR) treated chondrosarcoma bearing rats (*= $p < 0,05$; **= $p < 0,005$; ***= $p < 0,0005$). Data are given as the mean of each marker measured \pm SE. All values are in pg/ml; $n=6$ /group. Ctr: progressive chondrosarcoma; Chs PI3K: GDC-0941-treated SRC; Chs Akt: G-594-treated SRC; Chs mTOR: Everolimus-treated SRC

6.3.4 Take home message

- Inhibitors of PI3K, Akt and mTOR significantly inhibit chondrosarcoma growth in SRC model.
- The three inhibitors did not affect tumor immune infiltration.
- The cytotoxic assay and the seric cytokines level showed that the PI3K pathway inhibitors caused a decrease in the immune response toward chondrosarcoma.
- The immunosuppressive effects of PI3K pathway inhibitors abolished the idea to combine an agent targeting this pathway with immunotherapy.

6.4 Immune Checkpoint targeting

As they may also allow immune tolerance to tumors, immune checkpoint inhibitors against cytotoxic T lymphocyte antigen 4 (CTLA-4) or programmed cell death protein 1 (PD-1) or PD-1 Ligand (PD-L1) are showing promise in the clinic. They increase endogenous antitumor activity and might increase the tumour immunogenicity that is induced by chemotherapy, radiotherapy or targeted therapies. So there are opportunities to combine immune checkpoint inhibitors with first-line therapies.

Thus in a first step we evaluated the expression of the immunosuppressive ligand PD-L1 and its receptor PD-1 by tumor cells and tumor infiltrating cells were assessed both in human chondrosarcoma samples and in SRC. Then we tested the possibilities to modulate and/or block this immune checkpoint.

6.4.1 Expression of PD-1/PD-L1 in chondrosarcoma

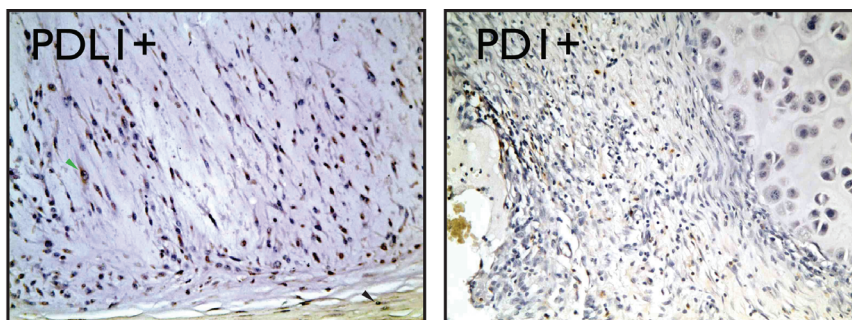
A) Conventional human chondrosarcoma

PD-L1 expression was evaluated in the same series of 26 human conventional chondrosarcoma presented in paragraph 6.1.

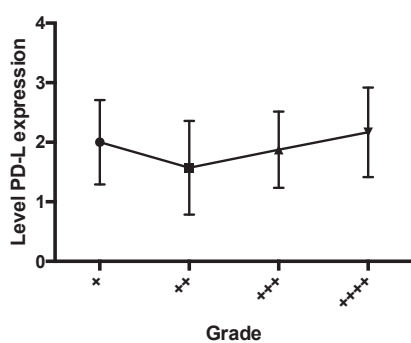
In this series, we found that PD-L1 was expressed in all 26 human chondrosarcoma tumors both on tumor cells and on tumor-infiltrating immune cells (Fig 6.20a, Table 6.1). PD-L1 expression differed among patients and was not correlated with tumor aggressiveness (Table 6.1 Fig 6.20B) but tended to be correlated with patient's survival and relapse: a higher expression of PD-L1 was associated with better survival and fewer relapse (Fig 6.20C). These results need to be confirmed on a larger cohort.

PD-1 was expressed in all human chondrosarcoma and its expression did not significantly vary among patients and was not associated with higher immune infiltrates or with superior PD-L1 expression (Fig 6.20a).

A



B



C

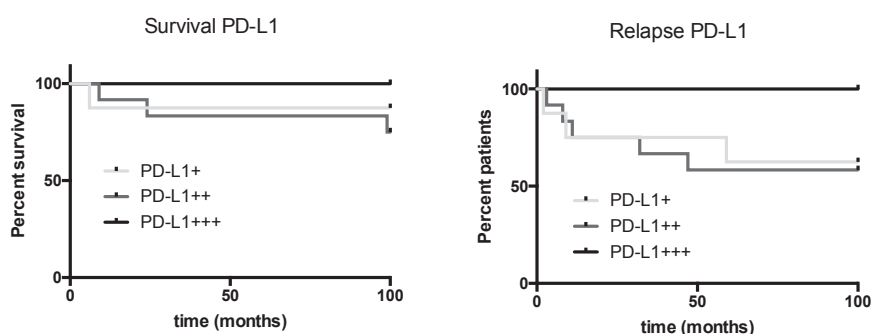


Figure 6.20: Expression of PD-L1 and PD-1 in human conventional chondrosarcoma. A) Representative IHC of PD-L1 and PD-1 expression in human chondrosarcoma showing diffuse membranous PD-L1 expression by tumor cells (green arrow) and tumor infiltrating immune cells (black arrow). PD-1 is highly expressed on tumor infiltrating lymphocytes (black arrow). B) Correlation between PD-L1 and tumor aggressiveness C) Correlation between PD-L1 and PD-1 expression and survival and relapse.

B) SRC

As in human chondrosarcoma, PD-L1 was detected both on tumor cells and on tumor infiltrating immune cells in SRC model while PD-1 was identified on tumor infiltrating immune cells (Fig 6.21).

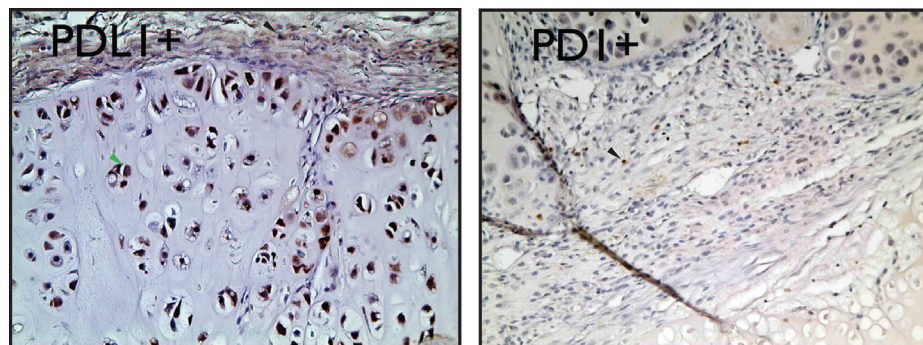


Figure 6.21: Expression of PD-L1 and PD-1 in SRC. Representative IHC of PD-L1 and PD-1 expression in rat chondrosarcoma. Tumor cells and tumor infiltrating immune cells showed an intense staining corresponding to a high expression of PD-L1 (green arrow), whereas PD1 expression was found on the membrane of tumor infiltrating lymphocytes (black arrow).

This pattern of expression associated with the other characteristics of the SRC model (tumor progression, immunological tumor microenvironment) validates it as accurate preclinical model for chondrosarcoma immunological therapeutics approaches.

6.4.2 Modulation of PD1/PDL1 expression in SRC

A) By targeted therapies

We analyzed the impact of PI3K pathway inhibitors (GDC-0941: PI3K inhibitor; G-594: Akt inhibitor and Everolimus: mTOR inhibitor) on PD-L1 and PD-1 expression. Surprisingly, all three inhibitors caused a decrease in PD-L1 expression. Fewer PD-L1 positive cells were found in these groups of treatment and the expression level of PD-L1 was also lower than in the normal progressive

SRC (Fig 6.22). Interestingly none of the PI3K pathway treatments affect PD-1 expression.

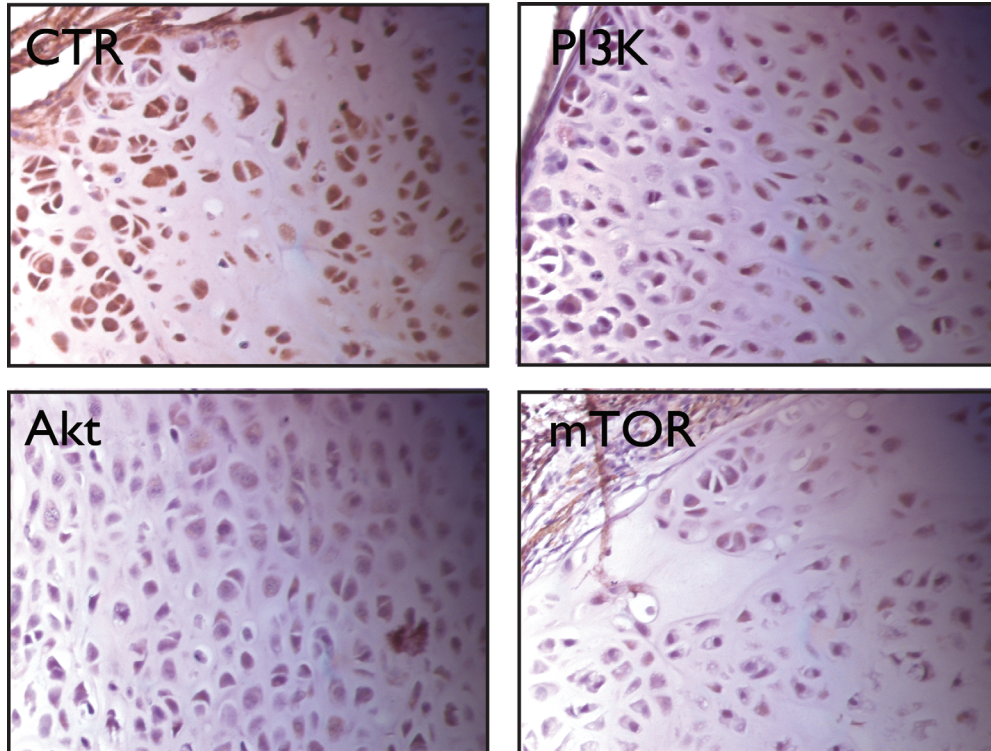


Figure 6.22: Expression of PD-L1 and PD-1 in PI3K pathway inhibitors treated SRC. Representative IHC of PD-L1 and PD-1 expression in SRC model after 3 weeks of PI3K pathway inhibitor treatment. A decrease in PDL1 expression was observed for all treatments.

This *in vivo* data were further confirmed *in vitro* in sarcoma cell lines in which expression of PD-L1 was stimulated by IFN γ .

Immunoblotting assays showed that PD-L1 expression is induced by IFN γ in all sarcoma cell lines used (Chondrosarcoma SW1353; osteosarcoma MNNG/HOS and rhabdomyosarcoma: RMS). Incubation of the three cell lines with PI3K pathway inhibitors, caused a net decrease in the expression of PD-L1, confirming the observation done *in vivo* in the SRC model (Fig 6.23).

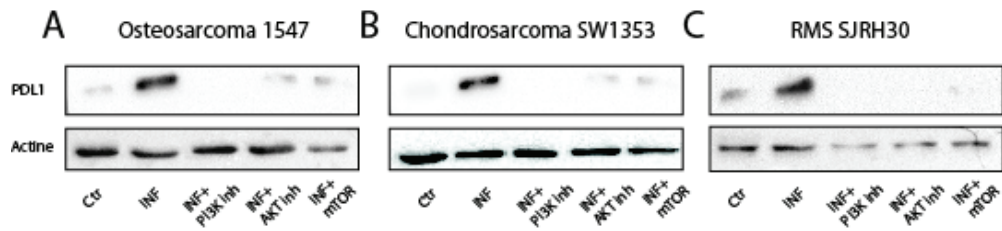


Figure 6.23: Expression of PD-L1 and PD-1 in PI3K pathway inhibitors treated sarcoma cell lines MNNG/HOS (osteosarcoma), SW-1353 (chondrosarcoma), RMS SJRH30 (Rhabdomyosarcoma) were treated by $\text{INF}\gamma$ known to upregulate PDL1 expression, then submitted to either PI3K, Akt or mTOR inhibitors (RAD) and expression of PDL1 was then analyzed by western blot (n=3)
 PI3K inh: GDC-0941; Akt inh: G-594; mTOR inh: Everolimus

These results indicate that PD-L1 is expressed on human chondrosarcoma cells, in the SRC model and sarcoma cell lines. We observe PD-1 expression on infiltrating cells both in human and in SRC. Moreover, our data show that PI3K/Akt/mTOR inhibitors downregulate PD-L1 expression in SRC and sarcoma cell lines. The next section will investigate further on the role of PD-L1 in chondrosarcoma.

B) By blocking antibody

To further investigate the role of PD-1/PD-L1 checkpoint in chondrosarcoma, it is necessary to block the interaction of PD-L1 with its receptor PD-1 *in vivo* and study the consequences of this blocking on tumor progression and immune environment. Nevertheless, there is a huge pitfall. No PD-L1 blocking antibody crossreacting with the rat species is yet available.

Therefore we examined the possibility to block rats PD-L1 using a murine PD-L1 blocking antibody. Expression of PD-L1 was induced by $\text{INF}\gamma$ stimulation in rat osteosarcoma cell line UMR-106. This expression was detected by flow cytometry with PD-1fc chimera (Fig 5.24 B). When pre-treated with PD-L1 blocking antibody, this expression was no longer detected (Fig 6.24)

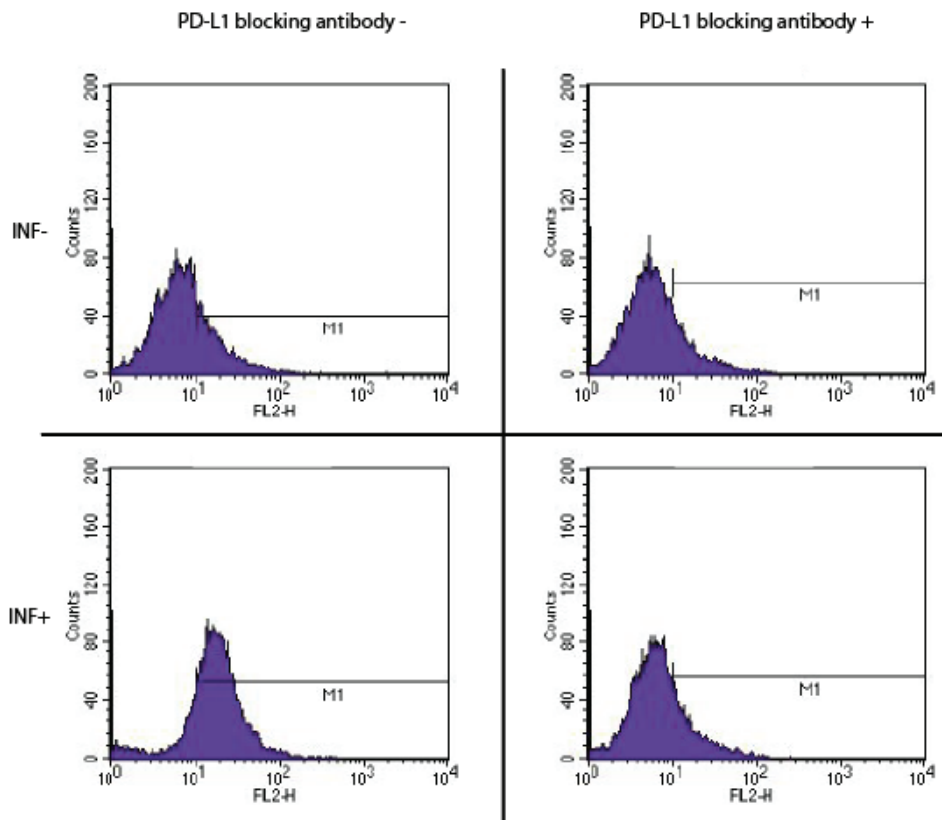


Figure 6.24: Blocking of rat PD-1 immune checkpoint using a murine PD-L1 blocking antibody. Expression of PD-L1 by rat UMR-106 osteosarcoma cell line is detected by flow cytometry using a murine PD-1Fc chimera coupled to FITC dye. Cells were pre-treated with $\text{INF}\gamma$ (bottom part of the quadrant) or not (upper part of the quadrant) and with PD-L1 blocking antibody (right side of the quadrant) or the isotype control (left side of the quadrant).

These results indicate that a murine PD-L1 antibody could be used in the SRC model to evaluate the effect of PD-L1 blocking strategy on chondrosarcoma progression. These assays are currently ongoing.

6.4.3 Take home message

- PD-L1 is expressed at different level in human chondrosarcoma patients, in SRC and in human sarcoma cell lines.
- PD-1 is expressed on tumor infiltrating cells both in human and SRC.

- PD-L1 expression is down regulated by the inhibitors of the PI3K/Akt/mTOR pathway inhibitors.
- Murine PD-L1 blocking antibody can block rat PD-1 protein.

7. Discussion

Chondrosarcomas (CHS) is a rare and heterogeneous family of mesenchymal tumors, accounting for 20% of bone malignancies, whose treatment has remained largely unchanged over the last 3 decades (4). 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 (4). However, cytokines and chemokines produced by CHS can cross the barrier formed by the extracellular matrix (ECM) (4), attract immune cells and can induce the secretion of metalloproteases which degrade the ECM a step essential for CHS invasion (232).

As new therapeutic approaches are being preclinically evaluated for chondrosarcoma, the hypothesis emerges that the best approaches could probably be to take advantages of phenotypic microenvironmental features of CHS instead of fighting them. Chondrosarcoma's immune microenvironment is one of the least known microenvironmental features of this tumor. Given the hopes and results generated by immunotherapies based on immune checkpoint inhibitors in different tumors types, it seems crucial for chondrosarcoma clinical management to assess the potential of such strategies. Thus, the aim of this study was: i) to describe the immune cell infiltrates associated with chondrosarcoma in human samples and in a syngeneic rat model recapitulating the human disease; ii) assess their functional role in CHS progression; iii) analyze the effects of targeted therapy on chondrosarcoma's immune environment and iv) determine the role of the PD1/PDL1 immune checkpoint on chondrosarcoma progression and evaluate the possibility to use blocking antibody anti-PDL1 in chondrosarcoma.

In this section of the manuscript, we will discuss of the prognostic value of T lymphocytes and macrophages infiltrates in chondrosarcoma, the opposite role of CD8+ and CD163+ populations in chondrosarcoma, the immunogenicity of chondrosarcoma cells, the immunomodulation of chondrosarcoma.

7.1 Prognosis value of T lymphocytes infiltrates in chondrosarcoma

As it was highlighted in the introduction of this manuscript, lymphocytes play an important role in the progression and control of cancer (142). Notably they are essential in the mechanism of tumor immunosurveillance (142).

So far, no study reporting the presence and implication of this immune population in chondrosarcoma microenvironment has been published. The following paragraphs resume and discuss our data relevant to T lymphocytes localization, implication and potential prognosis value for chondrosarcoma.

7.1.1 T lymphocytes in human chondrosarcoma

Our study was conducted on a series of 26 human conventional chondrosarcoma. In this series, we report the presence of T lymphocytes located in the marginal area of tumor on human CHS samples. This I infiltration is composed of CD3+, CD4+ and CD8+ T cells. We show that the infiltration of CD8+ T cells is a marker of good prognostic (section 6.1.3).

Indeed, patients with above the mean CD4+, CD8+ and CD3+ T cells infiltrates had better progression free survival than patients with below the median T cells counts. We also find that CD8+ T cells infiltration is correlated with the size of the tumor at the time of surgery (section 6.1.3). ; a high infiltration of CD8+ T cells being encountered in smaller tumor whereas a low CD8+ T cells count was associated with tumors of higher aggressivity infiltrating the surrounding tissues.

These data established on a small cohort of chondrosarcoma need to be conformed on a larger series of CHS. Nevertheless, they suggest that like for other types of cancer (melanoma, osteosarcoma and pleural mesothelioma), CD8+T cells infiltrations could be a prognosis factors for CHS outcome(233-235).

To fully elucidate the implication of T lymphocytes in chondrosarcoma, the CD4+ T cells, more particularly the Treg subset need to be studied. By gaining insight

into the CD4+ T cells infiltration and its potential correlation with chondrosarcoma patients evolution we will then be able to have a full picture of T cell's role in chondrosarcoma.

7.1.2 T lymphocytes in SRC model: immunological microenvironment in SRC is similar to human immunological microenvironment

Chondrosarcoma is a rare tumor and few biological materials are available to further study the implication of immune populations in this pathology and a relevant animal model is necessary to address this question. The Swarm rat chondrosarcoma (SRC) model is well suited for such studies as; unlike most sarcoma models established in immunocompromised animals, (236, 237) the tumors are established on immunocompetent animals, and grow orthotopically.

In the SRC model, the same immune populations (CD3+, CD8+ T lymphocytes) were identified in the same location as in the human samples. As in human conventional chondrosarcoma, a high CD8+ T cells infiltration is correlated with less aggressive tumor (as indicated by the measure of the tumor proliferative rate). These observations indicate that SRC model is accurate to study chondrosarcoma immunological environment. Thus further analyses conducted in SRC to gain insight into the implication of immune populations in chondrosarcoma could be translated to human chondrosarcoma.

The CD4+ T cells populations could not be analyzed by IHC staining in rats, as no reliable marker is yet available.

7.1.3 Impact of CD3+ and CD8+ T lymphocytes on chondrosarcoma progression in SRC model

Azathioprine, an agent blocking the proliferation of lymphocytes and preventing the differentiation of naïve and memory T cells in effectors T cells (230), was used to perform T cell depletion in SRC. Azathioprine caused a net decrease in T cells infiltrating SRC. This decrease was associated with a significant accrual in

tumor progression and a dramatic decrease in animal's survival ($P < 0,0005$). These data further support the implication of CD8+ T cells as agents restraining tumor growth.

Azathioprine did not solely inhibits CD8+ T cells; it affects all lymphocytes populations. Thus to gain further indications into the exact implication of each lymphocytic population on chondrosarcoma progression selective depletion using CD3 or CD8 blocking antibodies should be done. Those experiments are quite expensive to complete in rat models.

The use of azathioprine as depletion agent could be discussed as in addition to its immunosuppressive action this agent was initially develop as chemotherapy but didn't appear to have antitumor efficacy (238, 239). To date, the exact mechanisms of action of azathioprine in promoting cancer progression as not been reported but from our observations we could hypothesize that the decrease this agent causes in T cells will result in a decrease of immunosurveillance mechanisms which in turn will promote tumor progression.

One point we should take into consideration is the possible role of B lymphocytes, as their implication in tumor progression is more and more documented (240-242). The lack of reagents available for rat studies prevented us from studying this immune population.

7.2 Prognosis value of macrophages in chondrosarcoma

Tumor-associated macrophages (TAMs) have a dual role in cancer depending on their pro-inflammatory (M1) or pro-tumoral (M2) phenotype (243, 244). Generally M2 macrophages secrete anti-inflammatory cytokines (IL-10, TGF- β) and are mainly considered pro-tumoral (243). M1 macrophages secrete pro-inflammatory cytokines (TNF- α , IL-12) and are of good prognostic in certain tumors (244), even though a study showed that both M1 and M2 TAMS could be of good prognostic in osteosarcoma (243).

The following paragraphs resume and discuss our data relevant to macrophages localization, implication and potential prognosis value for chondrosarcoma.

7.2.1 Macrophages infiltrates in human chondrosarcoma

In the series of 26 human conventional chondrosarcoma, we reported the presence at the peripheral area of the tumor of macrophages expressing the CD163 antigen in all patients. In contrast to T lymphocytes infiltration, no significant correlation was found between CD163+ cells infiltration and overall survival or relapse of CHS patients (section 6.1).

However, CD163+ cells infiltration was correlated with tumor size and aggressivity, a higher CD163+ cells infiltration being associated with bigger tumors at the time of surgery and with tumors infiltrating surrounding tissues.

All these data suggest that CD163+ macrophages play a pro-tumoral role in human conventional chondrosarcoma.

These data established on a small cohort of chondrosarcoma need to be confirmed on a larger series of chondrosarcoma. Nevertheless, they suggest that like for other types of cancer (melanoma, osteosarcoma and pleural mesothelioma), CD163+ cells infiltrations could be a prognosis factor for CHS outcome(233-235).

This observation suggesting that in chondrosarcoma CD163+ TAMs role is pro-tumoral is similar to other made in melanoma or breast cancer (11). Surprisingly, these data differ from TAM activity reported in osteosarcoma (91), in which TAM are of good prognosis.

Like for the observation made on lymphocytes population, our data on macrophages infiltration are still incomplete.

The CD163+ maker we used is expressed by all macrophagic populations M1 and M2, even though its expression is higher on M2 population. Thus we can

conclude that TAMs in general and not just M2 are present in chondrosarcoma microenvironment and are involved in this tumor's progression (245).

We need to investigate the role of each M1 and M2 subpopulation in chondrosarcoma. This could be done on a larger cohort of chondrosarcoma patients using specific markers for M1 (CD14/HLA-DR α) and M2 (CD14/CD163) TAMs as it was done in osteosarcoma (91).

7.2.2 Macrophages infiltrates in SRC model

The implication of macrophages in chondrosarcoma was further evaluated in SRC model.

Similarly as in human chondrosarcoma an infiltration of CD163+ macrophages located in the marginal area of tumor was found in SRC. Like in human a high CD163+ infiltration correlated with the slowest growing tumor. Of note: CD163+ is actually the sole antibody available to identify macrophages in paraffin embeds rats tissues.

7.2.3 Effect of macrophages modulation on tumor progression

In our study, two agents were used to modulate macrophage population: i) clodronate which induces macrophage's death by apoptosis (231); ii) mifamurtide, a specific ligand of NOD2, which causes M2 to M1 macrophages conversion (203).

Clodronate caused a significant decrease in CD163+ cells associated with a significantly increased survival of the animals (P <0,05) (section 6.2.3). Mifamurtide also reduced the density of CD163+ cells in chondrosarcoma microenvironment, slowed down tumor progression and consequently improved animal's survival. These data confirm that phagocytic immune cells and mainly CD163+ macrophages play a role in chondrosarcoma progression, and can be modulated to induce tumor regression.

Mifamurtide has already been investigated in other bone tumor. This agent has proven effective in osteogenic sarcoma, such that regulatory authorities have approved the non-specific immune stimulator MTP (muramyl tripeptide) for human use in primary osteosarcoma (200). MTP activates macrophages and monocytes as a potent activator of immune response. It also improves the overall survival from 70 to 78% and results in a one-third reduction in the risk of death from osteosarcoma (200).

The choice of these two agents can be discussed:

- Clodronate does not specifically target macrophages, but causes apoptosis of all phagocytic populations (DC, neutrophils, monocytes) (246). Nevertheless, it was shown that in long-term depletion experimentation, similar to the one we conducted here, macrophages populations were the most affected by clodronate (246).

- Mifamurtide caused a net decrease in CD163+ macrophages infiltration without affecting the lymphocytes population. This agent did not cause depletion in macrophages but acts by favoring the conversion of M2 pro-tumoral macrophages to M1 pro-inflammatory macrophages (203). So we could say that mifamurtide had induce a M2 (CD163+) to M1 (CD163-) conversion in our model. The action of mifamurtide, the consequences of mifamurtide treatment on chondrosarcoma progression, indicates that M2 macrophages are normally present in progressive chondrosarcoma and that these M2 macrophages play a pro-tumoral role.

Nevertheless, this point needs to be confirmed by a clear identification of M1 population present in SRC microenvironment, point for which the tools are still lacking.

7.3 The opposite role of CD8+ and CD163+ populations in chondrosarcoma

As we have discussed in the previous sections, both CD8+ T cells and CD163+ populations play a role in the progression of chondrosarcoma, the first ones restraining it while the later ones promote it.

We also reported that the ratio CD8+ T cells/CD163+ cells are correlated with tumor aggressivity both in human chondrosarcoma and SRC model and that this ratio has a prognosis value in human chondrosarcoma. Indeed, a high CD8/CD163 ratio (i.e. more lymphocyte than macrophages) is correlated to better progression free survival, and smaller tumor at the time of surgery. A high CD8/CD163 is encountered in grade II non-invasive chondrosarcoma and I whereas a low CD8/CD163 ratio is found in grade II and III invasive tumors. This indicates that CD8/CD163 ratio could be used as a marker of tumor aggressivity as it is done in pleural mesothelioma (5).

The presence of each of these populations in chondrosarcoma environment is inversely proportional: indeed in the same sample concomitant high CD8+ T cells and high CD163+ infiltration could never be found. It seems like these two populations interfere with each other in term of recruitment to the tumor site. Given that the depletion of lymphocytes did not affect macrophagic composition of tumor environment and vice versa, it seems like these two populations do not directly interact. This indicates that another factor/element from the tumor microenvironment plays a role in inducing the recruitment of each population.

7.4 Immunogenicity of chondrosarcoma cells

Two elements indicate that chondrosarcoma induces an immune response and is susceptible to immunosurveillance:

-the fact that splenocytes isolated from chondrosarcoma-bearing rats have a specific cytotoxic action against chondrosarcoma cells while splenocytes from healthy rats didn't show any cytotoxic action.

-the increase of pro-inflammatory cytokines that was detected in sera of tumor bearing rats and absent in the sera of healthy animals.

In the splenocytes, two immune cells population could induce the specific lysis of the chondrosarcoma cells that we observe: The Natural Killer (NK) cells and the CD8 cytotoxic T Lymphocytes (CTL). We believe that if both populations are involved in the cytotoxic response we observed, this one is more a result of the action of CD8+ T cells.

Indeed, The Natural Killer (NK) cells belong to the innate immune cells. They are known to cause cell death to cells that have lost the expression of "self" proteins at their surface (majorly through the loss of MHC class I) (247). NK cells cannot be responsible of the totality of the cytotoxic action that we observe. Since we would also observe cytotoxicity with splenocytes coming from healthy rats.

We base this hypothesis on the fact that NK cells in the spleen are all on a naïve state of activation and they need only a danger signal (like the loss of MHC class I) at the surface of their target cell to induce their cytotoxic action (247). NK cells isolated from the spleen are all on a naïve state of activation and need a danger signal (like the loss of MHC class I) at the surface of their target cell to induce their cytotoxic action (247). So, if chondrosarcoma cells by themselves could generate a signal strong enough to activate NK cells we would observe cytotoxicity with splenocytes from healthy rats in co-culture with chondrosarcoma

cells. As we didn't observe such an activity we believe that if NK cells have a cytotoxic action in chondrosarcoma this activity is almost negligible.

The second population is the CD8 cytotoxic T Lymphocytes (CTL). In cancer, naïve CD8 lymphocytes must undergo maturation to become CTL expressing a TCR specific for a Tumor Associated Antigen (TAA) (248). These specific CTL are found not only in the microenvironment of the tumors but also in the spleen (248). The fact that splenocytes of progressive SRC have a specific cytotoxic action directed toward chondrosarcoma cells, makes us believe that CTLs present in the spleen of the chondrosarcoma bearing animals are responsible for this response. Another indication in favor of CTL implication in SRC immune surveillance is the decrease in cytotoxic action observed in azathioprine treated rats (i.e. in T lymphocytes depleted animals) (230).

The tumor-associated antigen against recognized by CTL still remains to be identified. It is known that normal chondrocytes can induce specific T lymphocytes response directed against membrane proteins that give rise to rheumatoid arthritis (249). Moreover, it was demonstrated that human chondrosarcoma express high level of diverse TAAs of the cancer-testis antigens (CTA) family (194). The expression of these CTAs in other sites than the testis gives rise to a strong immune response (108). These are two potential families of antigens which expression could be investigated in the SRC model. The identification of potential antigenic targets harbored by chondrosarcomas is currently one of the research program conducted by our group in association with the Immunosarc program consortium.

7.5 Immunomodulation and chondrosarcoma

7.5.1 Immunosuppressive action PI3K pathway inhibitors

In the course of our project, we demonstrated that inhibitors of the PI3K pathway affect chondrosarcoma progression and immune environment. These agents proved efficient in significantly slowing down tumor progression (section 6.3) as it was previously shown by our group for an mTOR inhibitor (77).

As we envisioned applying a combination of this targeted approaches with immune-modulatory strategy (PD1/PD-L1 blocking strategy), we first needed to analyse the effect of these inhibitors on chondrosarcoma immune response.

None of these inhibitors caused changes in immune population infiltration in chondrosarcoma. Moreover these agents caused a decrease in PD-L1 expression both *in vivo* and *in vitro*. By downregulating PD-1/PD-L1 on chondrosarcoma cells, the PI3K pathway inhibitors disrupt the checkpoint inhibitor that could enhance T cells response toward chondrosarcoma. Such an effect of PI3K pathway inhibitors (one Pan PI3K inhibitor, two dual PI3K/mTOR inhibitors and two mTOR inhibitors) was reported in glioblastoma (24). Authors discuss about the possibility to use these inhibitors in combination with a T cells activating immunotherapy. They also discuss on the possibility that the PI3K pathway inhibitor could have an immunosuppressive effect without confirming it.

One drawback of these inhibitors is the effect they have on the cytotoxic activity of splenocytes and on the pro-inflammatory seric cytokines. The three agents tested in our study caused a decrease in cytotoxic activity of SRC splenocytes associated with a decrease in the seric pro-inflammatory cytokines. These data suggest that these agents in addition to their anti-tumoral action have also an immunosuppressive activity.

These observations correlate with other studies which reported the immunosuppressive action of the PI3K/Akt/mTOR inhibitors (250). The authors

have shown that PI3K inhibitors and mTOR inhibitors affect both innate and adaptive immune response. In the end, it all conducted to a decrease of the immune response toward infected/cancerous cells (250).

The PI3K signaling pathway is involved in multiple processes, including cancer progression (251) and immune suppression (250). The implication of this pathway in these processes and the data we obtained raise questions regarding the use of PI3K pathway inhibitors in combination with immunotherapy.

On one hand, PI3K pathway inhibition would slow down chondrosarcoma progression and down regulate PD1/PDL1 immune checkpoint. On the other hand, PI3K pathway inhibitors could repress the cytotoxic immune response thus leading to a decrease in antitumor immune response.

7.5.2 Immune checkpoint in chondrosarcoma

Our data describing chondrosarcoma immune environment show that this tumor can be submitted to immunotherapeutic approaches, notably to the ones targeting immune checkpoints.

With all the observations we made, we thought that a vaccine therapy using cancer cells lysate therapy could be the most effective to induce a stronger immune response in chondrosarcoma. Unfortunately, such an approach was already tested in clinic with sarcoma and chondrosarcoma patients and didn't prove effective (194-196). This lack of effectiveness of vaccine therapy in chondrosarcoma could be related with expression of PD-L1 by this tumor. A strong PD-L1 expression can abolish a specific T cells response in cancer (178).

Chondrosarcoma have all the criteria to be reactive to an immunotherapy blocking the immunocheckpoint PD-1/PD-L1 (252):

- Chondrosarcoma express PD-L1.
- PD-1+ cells are present in the microenvironment of the tumors

- CD8+ T cells are presents in the microenvironment.

Moreover, strategies to reactivate antitumor immune responses with drugs targeting CTLA-4 and PD-1/PD-L1 have led to striking advances in the therapy of melanoma, some carcinomas, and hematopoietic neoplasia (252). For bone sarcoma, it has been shown that PD-1 is expressed on CTLs that infiltrate osteosarcoma and osteosarcoma cells express PD-L1 (211). However, very few papers report the beneficial effect of blocking PD-1/PD-L1 on osteosarcoma growth *in vivo* (253, 254). Thus we wanted to apply a PD-1/PD-L1 blocking strategy in SRC model.

Our biggest concern is that no PD-1 or PD-L1 blocking antibody crossreacting with the rat protein is yet available. Thus we had to built chimera to establish in *in vitro* cell models that a murine anti-PD-L1 antibody could block PD-1/PD-L1 interaction in rat bone sarcoma cell line.

Based on this interesting and promising data we are currently evaluating *in vivo* in the SRC model the possibility to block PD-1/PD-L1 by using this antibody. The consequences of PD-1/PD-L1 blocking on tumor progression and immune environment will be soon reported.

7.6 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 correlates with the outcome of the disease. The contribution of lymphocytes and macrophages to the tumor progression was demonstrated by selective depletion. We also show that chondrosarcoma could be susceptible to immunotherapy like PD-1/PD-L1 blocking antibody and mifamurdide. We also, observe that murine PD-L1 blocking antibody could be used *in vivo* in our SRC model to evaluate the

role of PD1/PD-L1 in chondrosarcoma and whether an immune checkpoint blocking strategy is relevant for chondrosarcoma.

We find that SRC is a perfect model to study immunotherapy for chondrosarcoma. The only problem remains the lack of tools available for the studies of the rat immune populations compared to what is available for the mouse. Our group is currently working on developing new tools (flow cytometry antibodies panels, detection of markers by qRT-PCR on microdissected tumors) to evaluate the immune response in the SRC model. This will allow better understanding of the immune microenvironment of chondrosarcoma and the evaluation of new therapeutic approaches in a model closely related to the human disease.

7.7 Future directions

- A more complete description of human chondrosarcoma environment is going to be performed in a large cohort of human chondrosarcoma.
 - To fully elucidate the implication of T lymphocytes in human chondrosarcoma, the CD4+ T cells, more particularly the Treg subset will be analyzed.
 - The possible role of B lymphocytes will also be addressed
 - M1 and M2 subpopulation will be better characterized using specific markers for M1 (CD14/HLA-DR α) and M2 (CD14/CD163) TAMs as it was done in osteosarcoma (91).
 - The identification of tumor associated antigens expressed in chondrosarcomas is also one of the research axes investigated by our team in a collaborative program (Immunosarc project)
- The rat SRC model will be used to:
 - Evaluate *in vivo* the possibility to block PD-1/PD-L1 by using murine PD-L1 blocking antibody as well as the consequences of

PD-1/PD-L1 blocking on tumor progression and immune environment.

- Clarify the impact of clodronate and mifamurtide on M1 macrophages. We are currently designing flow cytometry antibodies panel and q RT PCR primers to analyze M1 and M2 macrophages in tumor microenvironment.

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9. Annexes

9.1 Manuscript submitted

9.1.1 Letter of confirmation of the submission of the manuscript

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9.1.2 Manuscript

Title page

Title: Description of the immune microenvironment of chondrosarcoma and contribution to progression.

Authors list:

François A. Simard¹, Email : francois.simard@lyon.unicancer.fr

Iseulys Richert¹, Email : iseulys.richert@lyon.unicancer.fr

Alexandra Vandermoeten², Email : Alexandra.vandermoeten@univ-lyon1.fr

Anne-Valérie Decouvelaere³, Email : anne-valerie.decouvelaere@lyon.unicancer.fr

Jean-Philippe Michot³, Email : jean-philippe.michot@lyon.unicancer.fr

Christophe Caux¹, Email : christophe.caux@lyon.unicancer.fr

Jean-Yves Blay⁴, Email : jean-yves.blay@lyon.unicancer.fr

Aurélie Dutour¹, Email : aurelie.dutour@lyon.unicancer.fr

Affiliations list:

¹Cancer Research Center of Lyon, INSERM UMR 1052, CNRS UMR 5286, Centre Léon Berard, 28 rue Laennec, Lyon, France.

² SCAR, Université Claude Bernard Lyon1, Faculté de médecine et de pharmacie de Rockefeller, 8 Avenue de Rockefeller, Lyon, France.

³Pathology Department, Centre Leon Berard, 28 rue Laennec, Lyon, France.

⁴Medical Oncology Department, Centre Leon Berard, 28 rue Laennec, Lyon, France.

Full address for correspondence:

Aurélie Dutour, Cancer Research Center of Lyon, INSERM UMR 1052, CNRS UMR 5286, Bat. Cheney A Centre Leon Bérard, 28 rue Laennec, Lyon, France. Phone number: +33 4 78 78 29 32, email:aurelie.dutour@lyon.unicancer.fr

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Statement of Translational Relevance

New immune-targeted therapies have recently generated tremendous enthusiasm with the development of immune checkpoint inhibitors. As they may increase tumor immunogenicity induced by chemotherapy or targeted therapies, they seem to be of great interest for highly resistant tumors like chondrosarcoma. Chondrosarcoma immunological microenvironment remaining largely unknown, we designed a translational project aiming at describing it and deciphering its implication in chondrosarcoma growth and

identify potential therapeutic targets. We analyzed chondrosarcoma immunological microenvironment in human chondrosarcoma samples and in the Swarm rat chondrosarcoma model. We report here that CD8⁺ lymphocytes and CD163⁺ macrophages are involved in chondrosarcoma progression, and that PD-1/PD-L1 is expressed in chondrosarcoma. Our data indicate that immunomodulatory approaches can be envisioned for chondrosarcoma.

Abstract

BACKGROUND: Chondrosarcoma (CHS) is a rare bone malignancy characterized by its resistance to conventional systemic and radiation therapies. Whether immunotherapy targeting immune checkpoints like PD-1/PD-L1 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, macrophages and immune checkpoint expression in chondrosarcoma progression.

METHODS: Immunohistochemical stainings were performed on human chondrosarcoma samples and on Swarm rat chondrosarcoma model (SRC). Selective immunodepletion assays were performed in SRC to evaluate immune population's involvement in tumor progression.

RESULTS: 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). Expression of PD-1 and PD-L1 was detected in CHS immune infiltrates, both in human and rat (and on tumor cells). PD-L1 expression level correlated with patients survival and relapse rate. In SRC, selective depletions of T lymphocytes resulted in an accelerated growth rates, while CD163⁺ macrophages depletion 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%).

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

Key words

Chondrosarcoma, immune infiltrates, CD163 macrophages, PD-1/PD-L1, immune checkpoint.

Background

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 synthesize 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 still remains surgery with large en bloc tumor resection or amputation to ensure local control of tumor and to prevent future metastases (1, 4). Chondrosarcoma's 10-year survival rate range from 29–83% depending on CHS subtype and grade: novel therapeutic approaches are therefore urgently needed (1, 5, 6). cyclin-dependent kinase 4 (CDK4), matrix metalloproteinases (MMP), SRC, AKT, hypoxia-inducible factor (HIF), mTOR are possible relevant targets in chondrosarcoma (7-9). (10-13).

Little is known regarding chondrosarcoma microenvironment, besides its major proteoglycane (PG) contents and its hypoxic nature compared to 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 years (16), 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, while Treg and M2 macrophages may promote tumor progression in most cancers (17, 18), with the possible exception of metastatic osteosarcoma (19). The expression of PD-1 and its corresponding ligands (PD-L1 and PD-L2) are demonstrated mechanisms of tumor tolerance and escape. PD-1 is a cell surface receptor expressed on subsets of T and B lymphocytes, as well as other immune cells (20). In melanoma, ovarian and colorectal cancer PD-L1 is constitutively upregulated and the ligation of PD-1 by PD-L1 expressed on tumor cells inhibits CTL proliferation, cytokine production and cytotoxicity, thereby

leading to immune escape and tumor proliferation (21). The understanding of the role of immune checkpoints CTLA-4 and PD-1, and their therapeutical manipulation has led to novel immune-targeted therapies with major successes in diseases as various as metastatic malignant melanoma (MMM), non small cells lung cancer (NSCLC), bladder cancer, head and neck cancer (HNC) and Hodgkin's lymphoma (22).

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 (23) and the presence of M2 Tumor Associated Macrophages (TAM) is associated with metastases suppression (19). So far, one study has reported the expression of PD-1/PD-L1 in osteosarcoma samples (24) and PDL1-directed therapy in combination with CTLA-4 or PD-1 blocking antibody was efficient in a metastatic murine osteosarcoma model (25, 26).

In this study, we report on the description and role of immune infiltrates in chondrosarcoma. CD8⁺ T cells and CD163⁺ cells were observed at the margins of chondrosarcoma and their presence was found associated tumor's progression. We also show that the PD-1 is present at the surface of TIL, that PD-L1 is expressed by chondrosarcoma cells and TIL.

Materials and methods

Cell cultures

The human chondrosarcoma (SW1353), rhabdomyosarcoma (SJCRH30), bone marrow (K-562), Natural Killer (NK-92) cell lines, and rat osteosarcoma cell line (UMR-106) were all obtained from the ATCC (LGC Standards, Molsheim, France) 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 chondrosarcoma cells and chondrocytes were isolated from SRC or healthy animals by collagenase digestion (27) 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 Swarm rat chondrosarcoma model (SRC) has been previously described (28). This model is a grade II chondrosarcoma with mild cellular atypia. It mimics its human counterpart in terms of aggressivity and chemo resistance. Tumors were grafted on 25-days-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 periosteal abrasion. Upon establishment of palpable tumors, i.e. 10 days after tumor implantation, animals were randomly divided into treatments groups: PBS (1 ml; n=14) Azathioprine (2mg/kg; n=14; Sigma Aldrich, St-Louis, USA), Mifamurtide (0,1 mg/kg; n=14; Adooq bioscience, Irvine, USA), Clodronate (50 mg/kg; n=14; Clodronateliposomes.com, Amsterdam, The Netherland). Treatments were administered IP 5 days 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 2000 mm³. Tumor growth was monitored by regular visual inspection and tumor dimensions were measured every 2-3 days. Tumor volume was calculated using the formula:

Volume = longest tumor diameter x (shortest tumor diameter) ²/2. At the completion of treatment, rats were sacrificed, tumors and spleens harvested. Part of tumors and spleen were used for chondrosarcoma cells isolation and splenocytes harvesting while 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.

1.1.1 Splenocytes harvesting

Spleens were mashed on 40- μ m-cells strainer (BD Bioscience, San Jose, USA) placed on top of a 50 mL Falcon tube, using a syringe plunger. The collected cells were centrifuged (250 g, 5 min, 4°C) after addition of 10 mL RPMI medium supplemented by 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 (250 g, 5 min) and resuspended in complete RPMI medium.

Cytotoxicity assay

A nonradioactive, fluorometric cytotoxicity assay using calcein–acetoxymethyl (calcein AM; Thermofisher C3099) was performed to measure the lysis of rat chondrosarcoma cells and chondrocytes (further named target cells) by splenocytes. Target cells (1×10^6 /ml) were labeled with Calcein-AM (10 μ mol/L) at 37°C for 1 h before being washed twice in complete culture medium and plated at 10^4 cells/wells in round bottom 96-well microtiter plates (Nunc, **P8116**, Sigma Aldrich, St-Louis, USA). Target cells were then incubated with 400 μ mol/L Sulfinpyrazone (Sigma-Aldrich) in complete medium (1h, 37°C) to avoid spontaneous calcein release.

Splenocytes isolated from animals of all treatment group 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 6h 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 6h under normal culture conditions.

Supernatants were then collected (100 μ l) by centrifugation (250 g; 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 (29). For each sample, calcein release was measured on Tecan Infinite F500 (Tecan group ltd. Switzerland) (excitation filter: 485 ± 9 nm; band-pass filter: 530 ± 9 nm). Specific lysis induced by the effectors was calculated according to the formula:

Specific sample lysis= [(sample release – spontaneous release)/(maximal release – spontaneous release)] \times 100. All tests were run in triplicate.

1.1.2 Measurement of Cytokines secretion

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

1.1.3 Phenotypic analyses of chondrosarcoma immune microenvironment

Chondrosarcoma immune microenvironment was analyzed from 52 SRC and 26 conventional grade 1 to grade 3 human chondrosarcoma samples treated in our institution between 2004 and 2012. All samples were fixed in 4% formalin (pH 7.4) and embedded in paraffin. Deparaffination 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 μ m 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), PD-1 (1/100), PD-L1 (1/300) (respectively OX-8; ED2; AHP1706; AHP1703; AbD SeroTech, Oxford, UK). CD8 and CD163 primary antibodies were revealed using an unconjugated goat anti-mouse secondary antibody and CD3, PD-1, PD-L1 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 chondrosarcoma samples, 5 µm sections from FFPE blocks were deparaffinized and rehydrated. Immunostaining for CD3, CD8, CD4 and CD163 was performed on 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) (PD-1 and PD-L1 antibodies (AHP1706 and AHP1703 from ABD Serotec) were applied at 1/100. 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).

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

Quantitative analysis of chondrosarcoma immune infiltrates

Quantitative analysis of chondrosarcoma immune infiltrates was similarly conducted on SRC and human chondrosarcoma slides. Chondrosarcoma'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 to 25% of positive cells were encountered in the field; 2 when 26 to 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:

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

All the data are reported as the sample mean \pm the standard deviation (SD). Pairwise comparisons between means of different groups were performed using a Student 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 is considered statistically significant if the Student t-test gives a significance level (P value <0.05). Multiple comparisons were performed using an univariate analysis of variance (ANOVA).

Kaplan–Meier analysis was carried out 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 analysis was performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

Results

Chondrosarcoma immune microenvironment and tumor progression

The localization, lineage and density of immune cells present in chondrosarcoma microenvironment were analyzed in a cohort of 26 grade 1 to 3 conventional chondrosarcoma.

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 (Supplementary table S1). On each tumor, positive cells for each immune

marker were counted on 5 different areas located at the margin of cartilaginous nodules and surrounding muscles (magnification 200X) (fig 1a). The highest density of T cells was observed in invasive grade II and III chondrosarcoma.

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).

The density of CD163+ cells ranged from negligible to important (supplementary table S1) ; the highest density of CD163+ cells was found in grade III chondrosarcoma (Supplementary table S1). A correlation between high CD163+ cell count and tumor size was observed (Fig 2a). CD163+ infiltrations do not correlated with overall survival and relapse (Fig 1b,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 (30) count is inversely correlated with tumor size (Fig 2b) and tumor grade (Fig 2d). CD8/CD163 ratio, correlated to tumor grade (Supplementary table S2): the less aggressive tumors had the lowest CD8/CD163 ratio while G3 and G2 infiltrating tumors had the highest CD8/CD163 ratio. The same observation was made with CD8+ infiltration while the opposite effect was found with the CD163+ infiltration (Fig 2d).

SRC immune microenvironment recapitulates human chondrosarcoma

Immune infiltrates were then analyzed on rat chondrosarcoma from 17 untreated progressive SRC. As for human chondrosarcoma, 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 5 different areas located at the margin of cartilaginous nodules and surrounding muscles (magnification 200X) (fig 3a). In the SRC model the CD8/CD163 ratio and the CD8+ count were inversely

correlated with tumor size (Fig 3b,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 chondrosarcoma progression, selective depletions of T lymphocytes or macrophages were performed in SRC model.

Macrophages were targeted using either 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 clodronate or mifamurtide, a lower infiltration of CD163+ cells in tumor microenvironment was observed. In azathioprine treated animals, CD8+ and CD3 T cells were significantly decreased in tumor's environment (Fig 4a).

Both clodronate and mifamurtide affected tumor progression. Animals treated with these agents had significantly smaller tumors compared to control animals (respectively $1127 \pm 177 \text{ mm}^3$ and $1294 \pm 195 \text{ mm}^3$ versus $1913 \pm 202 \text{ mm}^3$ $P < 0,05$ on day 18) (Fig 4b). In rats treated with clodronate or mifamurtide, the tumor growth rate was lower than in the control group: tumor proliferation rate was respectively $16,23 \pm 2,19$ in the clodronate treated group and $24,32 \pm 3,42$ in the mifamurtide treated rats versus $63,17 \pm 14,32$ in the control group at d18 ($P < 0,005$) (Fig 4c). Both agents significantly delayed tumor progression and time necessary to reach the maximum tumor volume (2000 mm^3) (respectively $26,38 \pm 1,65$ days and $22,71 \pm 1,98$ days versus $16,92 \pm 0,70$ days in the

control group, $p < 0,05$) (Fig 4d). At day 34, 3 animals remained alive in the 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 ($91,90 \pm 21,48$ for azathioprine treated group versus $46,33 \pm 7,36$ for the control group $P < 0,05$ at day 14) (Fig 4c) with a significantly higher tumor volume than the control groups (respectively $2349 \pm 86 \text{ mm}^3$ versus $1434 \pm 169 \text{ mm}^3$ at d14 $P < 0,0005$) (Fig 4b). Azathioprine-treated rats reached the maximum tumor volume (2000 mm^3) sooner than the control group ($12,33 \pm 0,41$ days versus $16,92 \pm 0,70$ days $p < 0,0005$) (Fig 4d). At 14 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 pro-tumoral action while lymphocytes repress chondrosarcoma growth possibly by specific cytotoxic activity directed against chondrosarcoma cells. To elucidate this point, cytotoxicity of splenocytes from different treatment groups was analysed 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), clodronate-treated SRCs (n=6) and healthy animals (n=6) was evaluated against primary chondrosarcoma cells isolated from SRC tumors.

Splenocytes isolated from healthy animals did not have a cytotoxic activity against chondrosarcoma cells ((E:T ratio of 100:1, specific lysis of $2,5 \% \pm 1,7$). Conversely, chondrosarcoma cells were lysed by splenocytes isolated from chondrosarcoma bearing

rats: $(27,0 \pm 6,5\%$ was obtained at the E:T ratio 100:1). The splenocytes from azathioprine, treated rats had a lower cytotoxic activity on chondrosarcoma cells. $(11,3 \pm 3,5 \%$ 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 chondrosarcoma microenvironment and play an antitumoral role and maintain chondrosarcoma growth. Two key cytokines IFN γ and TNF- α were analyzed on all animal sera. IFN γ and TNF- α , were detectable in the sera of all chondrosarcoma animals while undetectable in healthy animals (Fig 5b,c,d). The three modulatory agents used (azathioprine, clodronate, mifamurtide) increased the level of seric IFN γ compared to untreated SRC animals ($P < 0,05$) (Fig5b,d). None of the immunomodulator treatments caused changes in TNF- α level (Fig 5c,d).

Expression of PD-1/PD-L1 in chondrosarcoma

Expression of the immunosuppressive ligand PD-L1 and its receptor PD-1 by tumor cells and tumor infiltrating cells were finally assessed both in human chondrosarcoma samples and in SRC. PD-L1 expression was observed in all 26 human chondrosarcoma both on tumor cells and on tumor-infiltrating immune cells (Fig 6a, Supplementary table S1). PD-1 was expressed on immune infiltrate cells in all human chondrosarcoma its expression did not significantly vary among patients and was not associated with higher immune infiltrates or with superior PD-L1 expression (Fig 6a, Supplementary table S1). The expression of PD-L1 and its receptor PD-1 were also found in SRC model. As in human chondrosarcoma, PD-L1 was detected both on tumor cells and on infiltrating immune cells (Fig 6b) when PD-1 was identified on tumor infiltrating immune cells (Fig 6b).

Discussion

Chondrosarcomas (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 3 decades (1). 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) (31). The aim of this study was to describe the immune cell infiltrates associated with chondrosarcoma, and determine their functional role in CHS progression, both in human samples and in a syngeneic rat model recapitulating the human disease.

Immune infiltrate composed of lymphocytes and macrophages were found located in the peritumoral area both on human CHS samples and in a rat chondrosarcoma 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 (32).

In a series of 26 samples, 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 chondrosarcoma. Similar observations, on the role of lymphocytes population, have been reported in other cancers (16) among which melanoma (33) and osteosarcoma (23).

No significant correlation was found between CD163+ infiltration and overall survival or relapse of CHS patient and was associated with biggest tumors. In osteosarcoma, tumor associated macrophages (TAM) (both M1 and M2 subtypes) are associated with a good prognostic compared to carcinomas where TAM are often considered to be pro-tumoral and are associated with bad prognosis (34). We also observed an inverse correlation

between CD163+TAM CD8+ lymphocytes infiltration, we 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 (35). These observations conducted in 26 chondrosarcoma patients need to be validated on a larger cohort.

Chondrosarcoma 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 Swarm rat chondrosarcoma (SRC) with the human counterpart, this model is well suited for such studies as; unlike in most sarcoma models established in immunocompromised animals, (36, 37) the tumors are established on immunocompetent animals, and grow orthotopically.

The SRC model mimics human grade II chondrosarcoma in term of growth and therapeutic resistance (38). 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 chondrosarcoma'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.

Azathioprine, an agent that blocks the proliferation of T cells and prevents the differentiation of naïve and memory T cells in effectors T cells (39) was used to perform T cell depletion in SRC. Azathioprine affected tumor'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 chondrosarcoma cells that is absent in azathioprine treated rats, indicating that T cells play an anti-tumoral role in this chondrosarcoma model, as in other tumor models (16).

M2 tumor associated macrophages (TAMs) (40, 41) has been shown to present a protumoral activity (18) (42). Clodronate which induces macrophage's death by apoptosis (43), 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 (44), reduced the density of CD163+ cells in chondrosarcoma microenvironment and resulted in an increase in animal's survival, confirming that phagocytic immune cells and mainly CD163+ macrophages may play a role in chondrosarcoma progression, and can be modulated to induce tumor regression. Mifamurtide, has already shown its potential therapeutic value in osteosarcoma (45).

In metastatic osteosarcoma model, blockade of PD-1/PD-L1 showed potent antimetastatic action (26). We evaluated the expression of PD-1/PD-L1 immune checkpoint both in human samples and SRC. PD-L1 was found expressed both in human samples and SRC model. Surprisingly, in human samples, high level of expression of PD-L1 was associated with an absence of relapse and death. Although non significant, the functional significance of this observation is being tested in the SRC model.

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 clodronate/mifamurtide in the SRC model.

Competing interests

All authors have declared no conflicts of interest.

Authors' contributions

FAS conceived the study, draft the manuscript and carried out every experiment.

IR and AV helped on *in vivo* and *in vitro* experimentation

AVD and JPM carried out 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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Figure legends

Figure 1: Immune infiltrate in human chondrosarcoma. A) From top to bottom representative IHC staining of CD8+, CD3+, CD4+, CD163+ cells (black arrow) in human chondrosarcoma (original magnification 200X). B) Immune populations are correlated with patients survival's. 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 based on the median of immune cell counts.

Figure 2: Impact of immune infiltrate on human chondrosarcoma progression. A) Correlation between CD8+cells (red dots), CD163+ cells (black dots) and tumor size. B) Correlation between the CD8/CD163 ratio and tumor size at the time of resection. C) Correlation between the infiltration of CD8+ lymphocytes and the CD163+ macrophages in the microenvironment of the tumors D) Correlation between CD8+ counts, CD163+ counts and CD8/CD163 ratio and tumor grade. (+ grade I chondrosarcoma; ++ non-invasive grade II chondrosarcoma; +++ invasive grade II chondrosarcoma; grade III chondrosarcoma)

Figure 3: Immune infiltrate in rat chondrosarcoma model. A) From top to bottom representative IHC staining of CD8⁺, CD3⁺, CD163⁺ (black arrow) from SRC (original magnification 400X). B) Correlation of CD8/CD163 ratio with tumor growth index measured at day 21 in progressive SRC. C) Correlation between CD8+cells (red dots), CD163+ cells (black dots) and tumor size at day 21 in progressive SRC.

Figure 4: Effects of selective depletion on tumor's immune environment. A) Average immune cells infiltration (CD3+, CD8+, CD163+) in each treatment groups at the end of the experimentation. Azathioprine caused a decrease in CD3 and CD8+ cells whereas mifamurtide and clodronate affected CD163+ cells. Counts were made on 5 areas / tumor (400X magnification). B) Tumor growth evolution in control and CD163 (mifamurtide or 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 clodronate treated) or CD3/CD8 (azathioprine treated) depleted chondrosarcoma (*= $p < 0,05$; ***= $p < 0,005$).

Figure 5: Cytotoxic and inflammatory responses in chondrosarcoma. A) Specific cytotoxicity of splenocytes isolated from tumor-bearing animals. Non tumor-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 (*** = $p < 0,0005$). B) Serum IFN γ levels in normal animals, progressive chondrosarcoma CD163 or CD3/CD8 depleted chondrosarcoma bearing rats (*= $p < 0,05$). C) Serum TNF- α 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 IFN γ , TNF- α 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).

Figure 6: Expression of PD-L1 and PD-1 in human and rat chondrosarcoma. A) Representative images of PD-L1 and PD-1 expression in human chondrosarcoma showing diffuse membranous PD-L1 expression by tumor cells (green arrow) and tumor infiltrating immune cells (black arrow). PD-1 is highly expressed on tumor infiltrating lymphocytes (black arrow). B) Representative figure of PD-L1 and PD-1 expression in rat chondrosarcoma. Tumor cells and tumor infiltrating immune cells showed an intense staining demonstrating a high expression of PD-L1 (green arrow) and on tumor infiltrating immune cells (black arrow), whereas PD1 expression was found on the membrane of tumor infiltrating lymphocytes (black arrow).

Figure 1

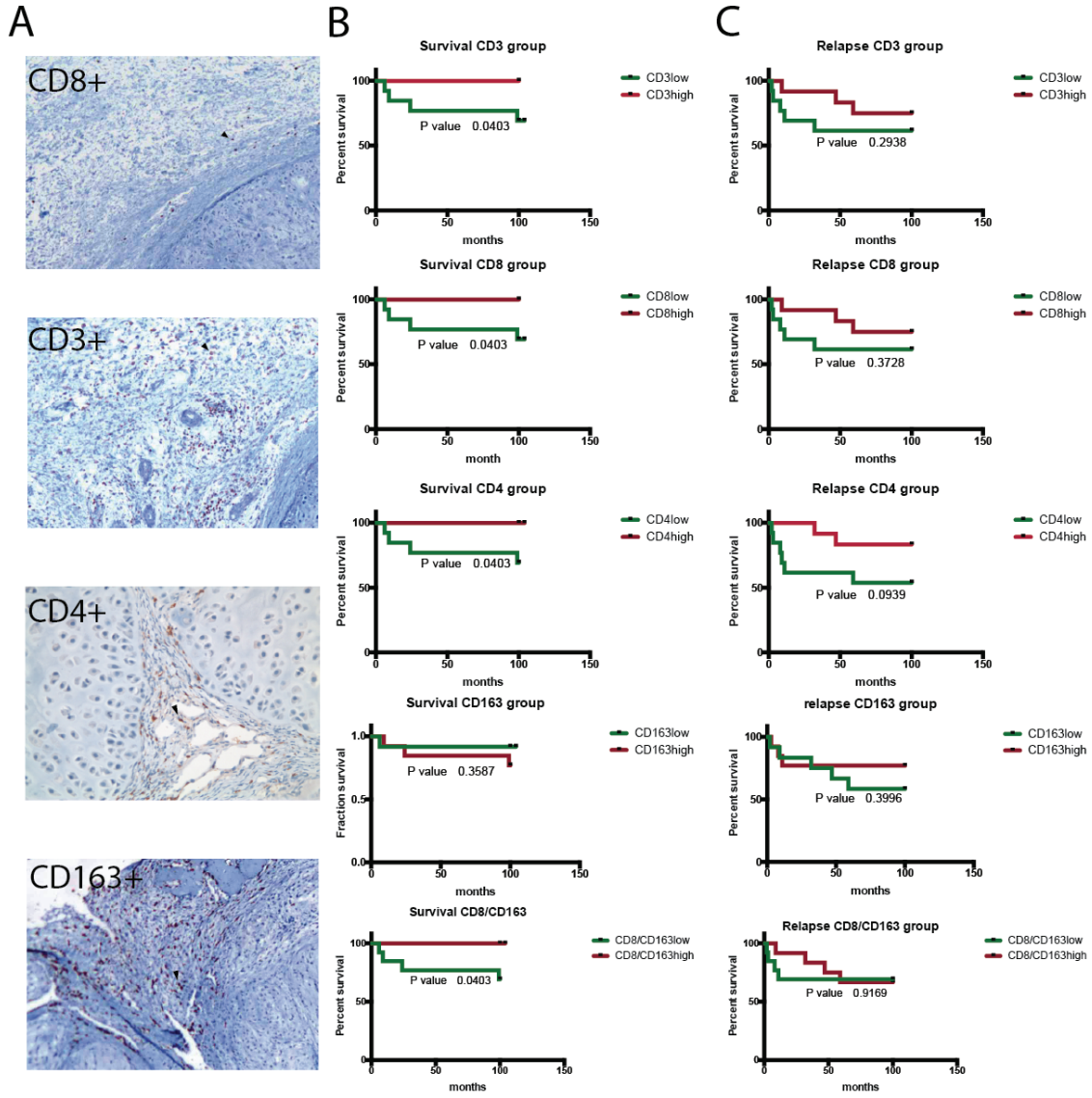


Figure 2

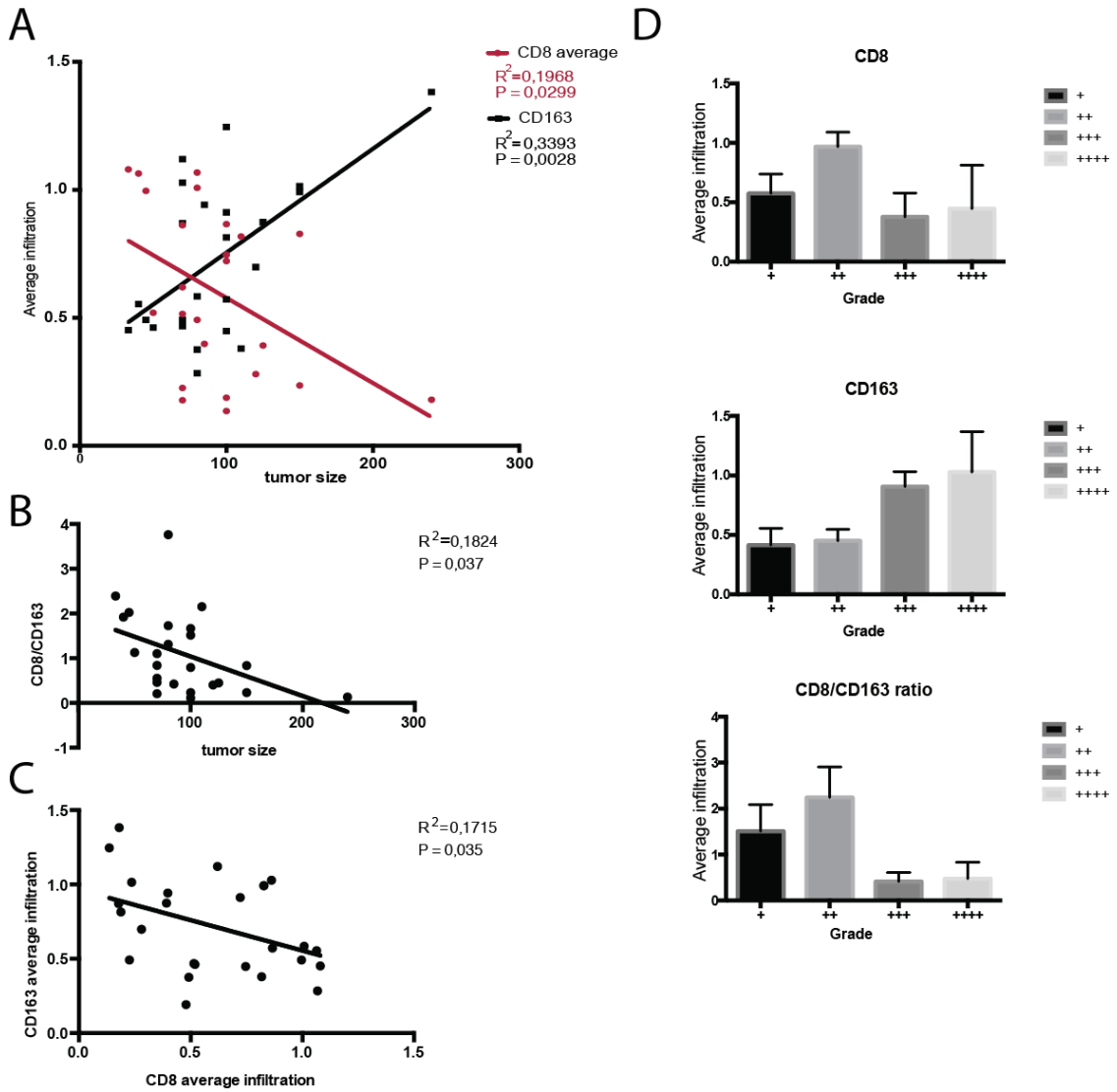


Figure 3

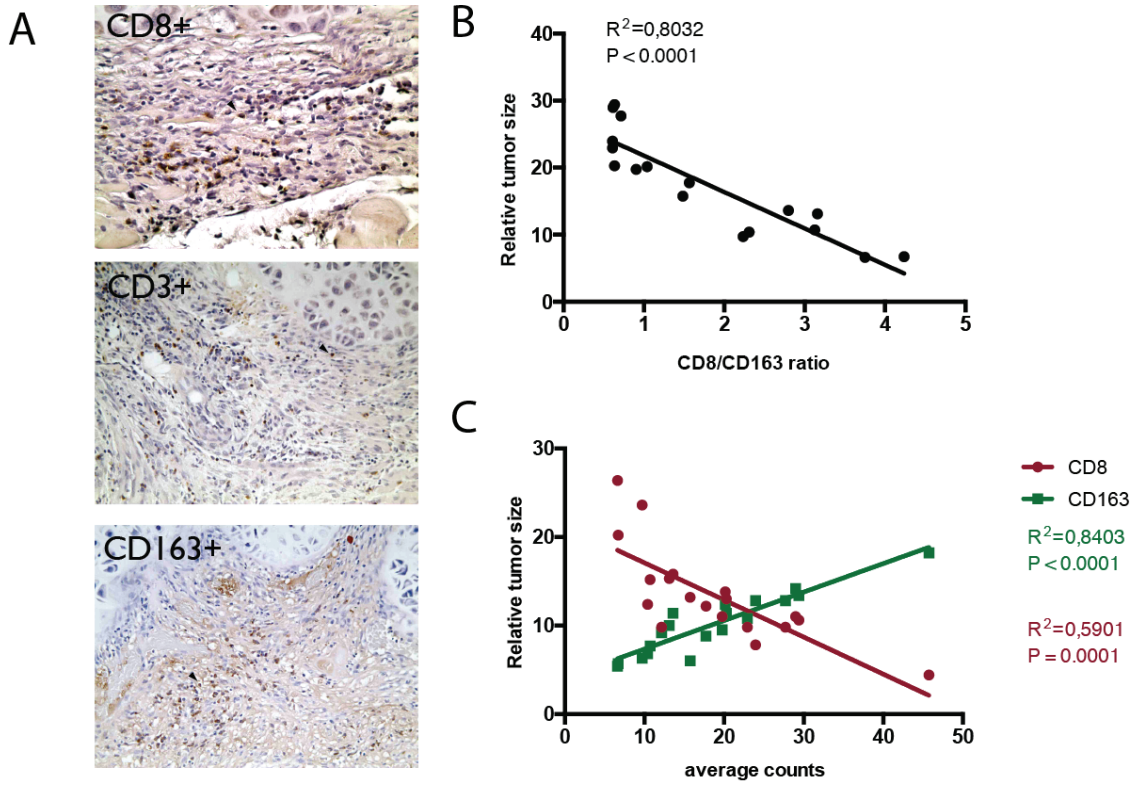


Figure 4

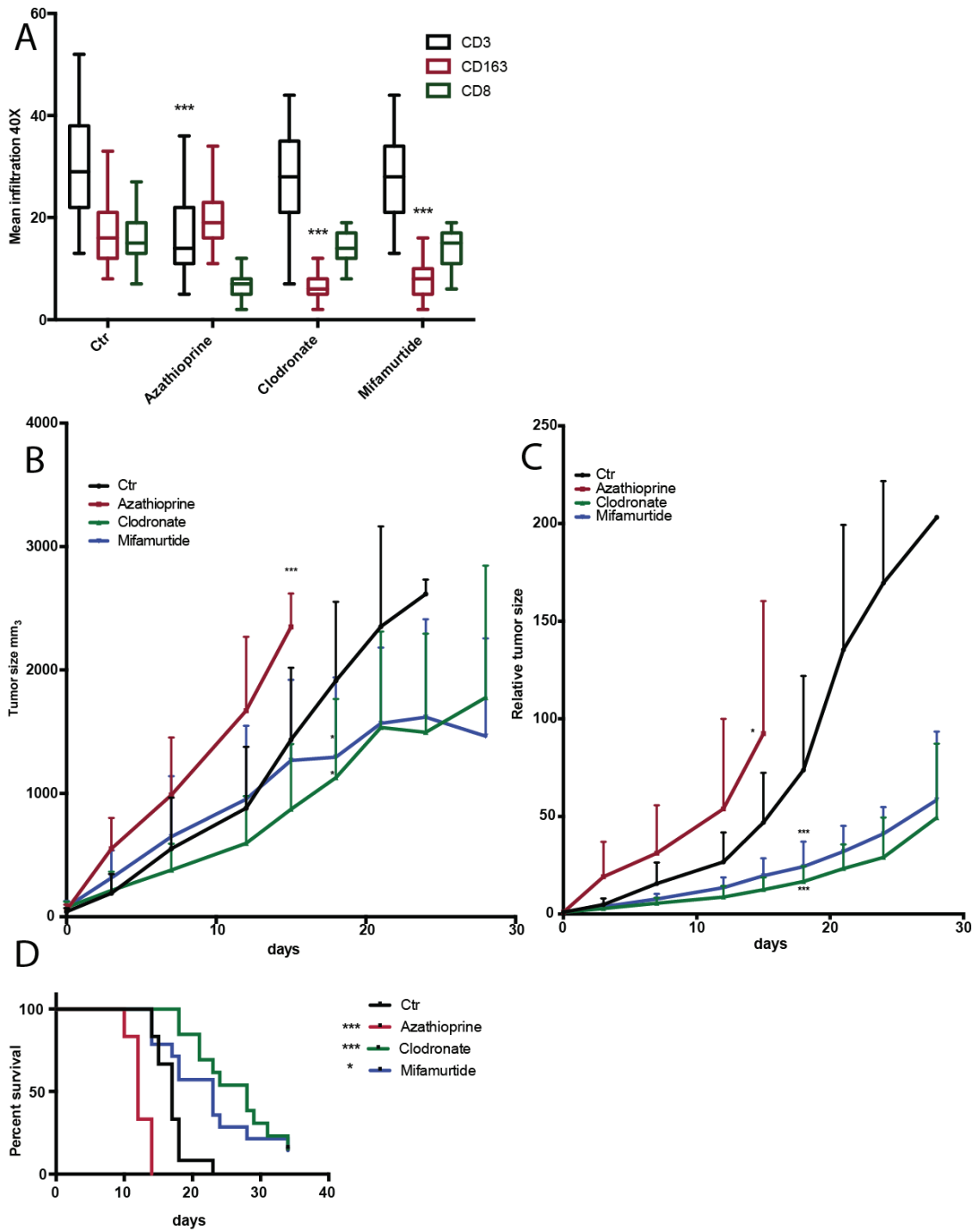
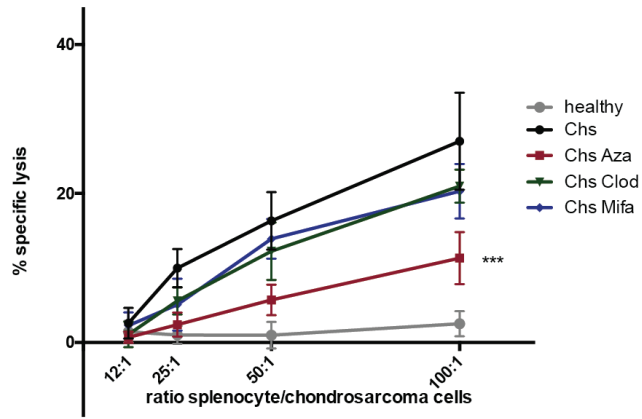
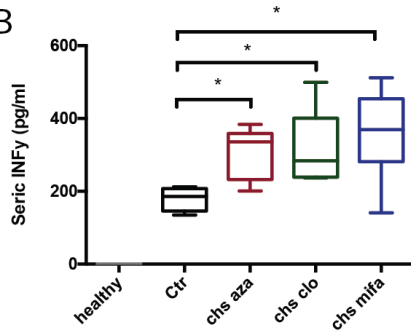


Figure 5

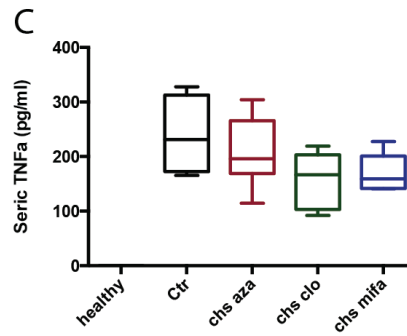
A



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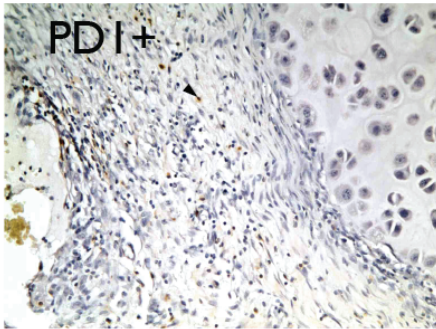
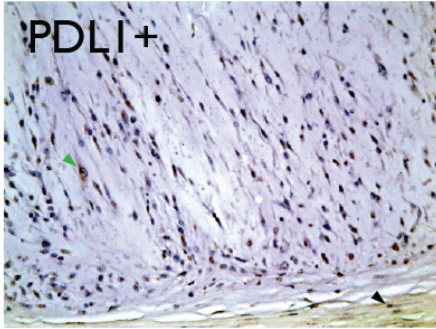


D

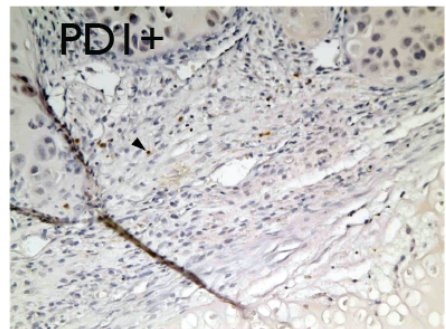
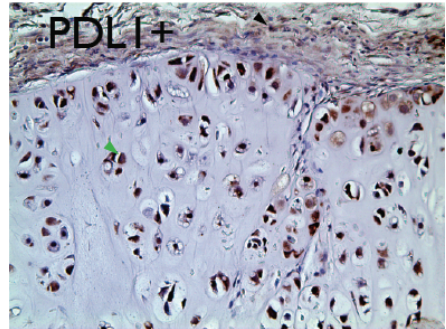
	INF- γ	TNF- α
Healthy	< 32	< 47
CTR	179,6 \pm 16,4	238,8 \pm 36,7
Azathioprine	299,6 \pm 28,3	206,8 \pm 23,7
Clodronate	318,5 \pm 41,4	159,3 \pm 18,4
Mifamurdide	351,0 \pm 51,0	169,5 \pm 12,3

Figure 6

A



B



9.2 Abstract for oral and poster presentation

9.2.1 Abstract for oral presentation Biosarc Paris 2015

“Implication of immune system in chondrosarcoma progression and therapeutic response. Could immunotherapy play a role in chondrosarcoma treatment?”

Simard François¹, Decouvelaere Anne-Valérie², Vandermoeten Alexandra³, Caux Christophe¹, Blay Jean-Yves⁴, Dutour Aurélie¹

¹ Department of Clinical Sciences, Centre de Recherche en Cancérologie UMR Inserm U1052-Equipe 11- CLB – Lyon, France

² Department of Biopathology and Cytology, Centre Léon Bérard, Lyon, France

³ Faculty of medicine Lyon Est, University Claude Bernard Lyon 1

⁴ Department of Medical Oncology, Centre Léon Bérard, Department of clinical sciences, Centre de Recherche en Cancérologie, Lyon, University Claude Bernard Lyon 1, Lyon, France

Chondrosarcoma is highly resistant to chemotherapy and radiation and there is an urgent need in developing new therapeutic strategies for this malignancy; among these, some immunotherapy approaches could be of great interest. We are currently investigating the immune system implication in chondrosarcoma progression and therapeutic response both on human samples and in rat chondrosarcoma model.

Macrophages (CD163⁺), lymphocytes (CD3⁺) and cytotoxic effector lymphocytes (CD8a⁺) were found in the microenvironment of chondrosarcoma both in human samples and in Swarm rat chondrosarcoma (SRC) model. We found out that there is a relationship between CD8a+/CD163 ratio and tumor progression. A low ratio is found in fast growing tumor and a high ratio in the slow growing ones. To understand their implication in chondrosarcoma growth, we used pharmaceutical agents to specifically target macrophage and lymphocyte populations. Azathioprine, an agent blocking the differentiation of effector T cells, caused an increase in tumor progression. On the opposite, clodronate, which caused a depletion of phagocytic macrophages, caused a significant decrease in chondrosarcoma progression. Additionally, we show in *in vitro* cytotoxic assays that splenocytes from chondrosarcoma bearing-rats present a cytotoxic action directed toward chondrosarcoma cells (35% at E:T ratio of 100:1) whereas splenocytes from healthy animals do not show this activity (2.5% at E:T ratio of 100:1). These results demonstrate the implication of immune infiltrates in chondrosarcoma progression and indicate that an immune response against chondrosarcoma cells takes place in the SRC.

The function of macrophages and cytotoxic immune cells in tumor microenvironment is being investigated and the expression of PDL1 in chondrosarcoma was reported here for the first time (PDL1 was found expressed in 100% of human samples (n=27) and rat chondrosarcoma model (n=52), as well as in bone sarcoma cell lines (n=9)). These results prompt us to focus on the implication of the immune infiltrate and PDL1 in chondrosarcoma progression and on the possibility to target PDL1 and the immune infiltrate as novel therapeutic approaches for chondrosarcoma.

9.2.2 Abstract for poster presentation 2015 CRCL symposium

“Implication of immune system in chondrosarcoma progression and therapeutic response. Could immunotherapy play a role in chondrosarcoma treatment?”

Simard François¹, Decouvelaere Anne-Valérie², Blay Jean-Yves³, Dutour Aurélie¹

¹ *Department of Clinical Sciences, Centre de Recherche en Cancérologie UMR Inserm U1052-Equipe 11- CLB – Lyon, France*

² *Department of Biopathology and Cytology, Centre Léon Bérard, Lyon, France*

³ *Department of Medical Oncology, Centre Léon Bérard, Department of clinical sciences, Centre de Recherche en Cancérologie, Lyon, University Claude Bernard Lyon 1, Lyon, France*

Chondrosarcoma is highly resistant to chemotherapy and radiation and there is an urgent need in developing new therapeutic strategies for this malignancy; among these, some immunotherapy approaches could be of great interest.

We are currently investigating the immune system implication in chondrosarcoma progression and therapeutic response both on human samples and in rat chondrosarcoma model.

Macrophages (CD163⁺), CD3⁺ lymphocytes and cytotoxic effectors lymphocytes (CD8a⁺) were found in the microenvironment of chondrosarcoma both in human samples and in the rat model. The ratio of CD8a⁺/CD163 change in function of the rat chondrosarcoma growth. A low ratio is found in fast growing tumor and a high ratio in the slow one, but we don't know if the infiltration provoke the growth change or if it's a consequence. We then use pharmaceutical agents to target the specific macrophages and lymphocyte population to reveal their role in the cancer growth. With azathioprine (Aza) that blocks the differentiation of naïve lymphocyte in effectors, we saw an increased of the tumor growth. By opposition, when we use clodronate liposome, we deplete phagocytic macrophage and see a significant decrease in the chondrosarcoma growth. Additionally, we show in *in vitro* cytotoxic assays that splenocytes from chondrosarcoma bearing-rats present a cytotoxic action directed toward chondrosarcoma cells (35% of lysed target cells) whereas splenocytes from healthy animals don't present this activity (2.5% of lysed target). These results demonstrate the possibility of applying immunotherapy for chondrosarcoma treatment. One approach that has recently emerged aims at targeting immune checkpoint that blocks the immune response to cancer. PD1/PDL1 is one of these immunecheckpoints which has already been shown to be a good target in different cancer models [2]. PDL1 was found expressed on 100% of human samples (n=27) and rat chondrosarcoma model (n=52), as well as in bone sarcoma cell lines (n=9).

The function of macrophages and cytotoxic immune cells in tumor microenvironment is being investigated and the expression of PDL1 in chondrosarcoma was reported here for the first time. These results prompt us to focus on the implication of the immune infiltrate and PDL1 in chondrosarcoma progression and on the possibility to target PDL1 and the immune infiltrate as novels therapeutics approach for chondrosarcoma.

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9.2.3 Abstract for poster presentation 2014 CTOS Berlin

“Implication of immune system in chondrosarcoma progression and therapeutic response. Could immunotherapy play a role in chondrosarcoma treatment?”

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¹ Department of Clinical Sciences, Centre de Recherche en Cancérologie UMR Inserm U1052-Equipe 11- CLB – Lyon, France

² Department of Biopathology and Cytology, Centre Léon Bérard, Lyon, France

³ Department of Medical Oncology, Centre Léon Bérard, Department of clinical sciences, Centre de Recherche en Cancérologie, Lyon, University Claude Bernard Lyon 1, Lyon, France

Context

Chondrosarcoma constitute a group of slow growing and heterogeneous tumors accounting for 20% of bone malignancies that resist to chemotherapy and radiations. The treatment of choice remains wide local excision when feasible. Thus the 10-year survival rate of chondrosarcoma has remained unchanged over the past 40 years [1]. Improving chondrosarcoma clinical management is a challenge and new therapeutic approaches are needed. New immunotherapy approaches are being developed, some of which could maybe benefit to chondrosarcoma.

Material and method:

Thus we are currently investigating the immune system implication in chondrosarcoma progression and therapeutic response; this project should enable the identification of immunotherapy targets.

Results

Our preliminary results show the presence by IHC of pro-inflammatory macrophages (CD163⁺), of lymphocytes (CD3⁺) and cytotoxic effectors lymphocytes (CD8a⁺) in the microenvironment of grade 2 chondrosarcoma both in human samples and in a rat chondrosarcoma model. Additionally, we show in *in vitro* cytotoxic assays that splenocytes from chondrosarcoma bearing-rats present a cytotoxic action directed toward chondrosarcoma cells (35% of lysed target cells at an effector/target ratio of 25) whereas splenocytes from healthy animals don't show such activity (2.5% of lysed target cells at the same ratio; n=2). These results demonstrate the possibility applying immunotherapy for chondrosarcoma treatment; one approach that has recently emerged and is of great interest aims at targeting immune checkpoint that blocks the immune response to cancer. PD1/PDL1 is one of these immunecheckpoints which has already been shown to be a good target in different cancer models [2]. PD-L1 was found expressed on 100% of human samples (n=8) and rat chondrosarcoma model (n=52), as well as in bone sarcoma cell lines (n= 9). *In vitro*, PD-L1 expression is induced 10-fold by INF γ and treatment with inhibitors of the PI3K/mTOR pathway downregulates its expression (n=6). Such results were also observed *in vivo* in animals receiving PI3K/mTOR pathway inhibitors (n=6).

Conclusion

These preliminary results prompt us to further investigate the implication of PD-L1 in chondrosarcoma progression as well as the possibility to target this protein as therapeutic approach for chondrosarcoma.

REFERENCES

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9.2.4 Abstract for oral presentation 2014 GSF-GETO La Turballe

"Immune and stromal responses in cancerology: new challenges for therapeutic targeting" Workshop
Pointe de Pen Bron in La Turballe, France, October 1st- 4th, 2014



"Implication of immune system in chondrosarcoma progression and therapeutic response. Could immunotherapy play a role in chondrosarcoma treatment?"

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¹ Department of Clinical Sciences, Centre de Recherche en Cancérologie UMR Inserm U1052-Equipe 11- CLB – Lyon, France

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Titre thèse français: L'implication du système immunitaire dans la progression et la réponse thérapeutique du chondrosarcome. Est-ce que l'immunothérapie peut jouer un rôle dans le traitement du chondrosarcome ?

Le chondrosarcome (CHS) est caractérisé par une grande chimio et radorésistance ; il y a un besoin urgent de nouvelles stratégies thérapeutiques pour cette tumeur. Parmi celles-ci, certaines approches d'immunothérapie pourraient être d'un grand intérêt. Nous étudions actuellement l'implication du système immunitaire dans la progression du CHS et la réponse thérapeutique à la fois sur des échantillons humains et dans le modèle de chondrosarcome de rat (SRC).

Dans le CHS humain et de rat, des infiltrats immunitaires composés de lymphocytes et macrophages ont été identifiés dans la zone péri-tumorale. L'infiltration immunitaire est en corrélation avec l'évolution de la tumeur (grade, envahissement et taille). L'expression de PD1 et PDL1 ont été détectée dans les infiltrats immunitaires et cellules tumorales du CHS chez l'homme et le rat. Le niveau d'expression PD-L1 est en corrélation avec la survie des patients et le taux de rechute. Dans le model SRC, la déplétion sélective de lymphocytes T a entraîné une accélération de la progression tumorale, tandis que la déplétion de macrophages l'a ralenti. Les splénocytes isolés de rats porteurs de CHS ont montré une cytotoxicité spécifique dirigée contre les cellules de chondrosarcome (27%), qui a diminué de manière significative avec des rats appauvrie en CD3 (11%). Les inhibiteurs de la voie de signalisation PI3K/mTOR ne peuvent pas être associés à une immunothérapie car ils induisent une action immunosuppressive *in vivo*.

L'environnement immunitaire contribue à la progression du CHS à la fois chez l'homme et chez le rat, ce qui suggère que une approche immunomodulatrice avec des anticorps bloquant PDL1 pourrait être testée pour le CHS.

Mots Clés : Chondrosarcome, lymphocyte, PD-1, PD-L1, macrophage, immunothérapie, PI3K/Akt/mTOR, immunosurveillance

Chondrosarcoma is highly resistant to chemotherapy and radiation and there is an urgent need in developing new therapeutic strategies for this malignancy; among these, some immunotherapy approaches could be of great interest. We are currently investigating the immune system implication in chondrosarcoma progression and therapeutic response both on human samples and in rat chondrosarcoma model (SRC).

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). Expression of PD-1 and PD-L1 was detected in CHS immune infiltrates, both in human and rat (and on tumor cells). PD-L1 expression level correlated with patients survival and relapse rate. In SRC, T lymphocytes depletion resulted in an accelerated tumor progression, while CD163+ macrophages depletion 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 PI3K/mTOR pathway inhibitors can't be associated to an immunotherapy since they induce an immunosuppressive action *in vivo*.

The immune environment contributes to CHS progression in both human and animal models, this associated with expression of immune checkpoint PD1/PDL1 suggest that immunomodulatory approaches with PD-L1 blocking antibody could be applied in CHS; this approach is currently being tested in SRC.

Keywords :Chondrosarcoma, lymphocyte, PD-1, PD-L1, macrophages, immunotherapy, PI3K/Akt/mTOR, immunosurveillance