The contribution of immune infiltrates and the local microenvironment in the pathogenesis of osteosarcoma

Marie-Françoise Heymann, Frédéric Lézot, Dominique Heymann

The contribution of immune infiltrates and the local microenvironment in the pathogenesis of osteosarcoma

HEYMANN Marie-Françoise[1,2], LÉZOT Frédéric [3,4] HEYMANN Dominique[1,2,4,*]

[1] Institut de Cancérologie de l'Ouest, site René Gauducheau, Boulevard Professeur Jacques Monod, 44805 Saint-Herblain, France.

[2] University of Sheffield, Department of Oncology and Metabolism, INSERM, European Associated Laboratory "Sarcoma Research Unit", Medical School, Beech Hill Road, S10 2RX, Sheffield, UK.

[3] INSERM, UMR1238 Team1, Faculty of Medicine, Nantes, France.

[4] University of Nantes, Faculty of Medicine, 44035 Nantes, France.

Key words: osteosarcoma, immune tolerance, tumour microenvironment, immunotherapy

*** Corresponding author**

Prof. Dominique Heymann
Institut de Cancérologie de l'Ouest,
Site René Gauducheau, Boulevard Professeur Jacques Monod,
44805 Saint-Herblain, France.

E-mail: dominique.heymann@univ-nantes.fr
ORCID: 0000-0001-7777-0669

Abstract

Osteosarcoma is a rare primary bone cancer characterized by cancer cells producing calcified osteoid extracellular matrix and inducing lung metastases with a high frequency. The local microenvironment defined several tumor niches controlling the tumor growth and cell extravasation. The immune infiltrate composes one of these niches. The immune environment of osteosarcoma is mainly composed by T-lymphocytes and macrophages but also contains other subpopulations including B-lymphocytes and mast cells. Osteosarcoma cells control the recruitment and differentiation of immune infiltrating cells and establish a local immune tolerant environment favorable to the tumor growth, drug resistance and the occurrence of metastases. Osteosarcoma cells are able to affect the balance between M1 and M2 macrophage subtypes and so could control the T-lymphocyte responses via the PD-1/PDL-1 system. In addition, mesenchymal stem cells may also contribute to this immune tolerance and strengthen the immune evasion. The present review gives a brief overview of the immune components of osteosarcoma and their most recent therapeutic interests.

Key words: osteosarcoma; immune tolerance; tumor-associated macrophage; tumor infiltrating lymphocyte; immunotherapy; microenvironment

Introduction

Osteosarcoma is the main primary bone malignant entity affecting adolescents and young adults [1,2]. Osteosarcomas are bone-forming tumors characterized by the presence of an extracellular osteoid matrix produced by cancer cells and associated with a very complex local environment including bone cells, blood vessels, stromal cells and immune infiltrates [3-6]. The osteosarcoma cell is the tumorous counterpart of osteoblast. This cell with a mesenchymal origin is the consequence of an initial oncogenic event altering for instance *p53* or *Rb* and occurring during the commitment of a mesenchymal cell toward osteoblast [7,8]. The oncogenic process leading to the establishment of an osteosarcoma is characterized by numerous complex events and requires multiple cellular partners. Based on genome sequencing analyses, a model of the natural history of osteosarcoma has been proposed recently [9]. These authors theorized that the disease may be initiated by a mutation of *TP53* and/or *Rb1* which show a very high recurrence in osteosarcoma. These cells that exhibit a high chromosomic instability lead to secondary genetic aberrations and to the emergence of new cancer cell clones from the initial monoclonal disease. The main consequence of all these events is the development of a complex disease characterized by a very high number of genetic alterations. Indeed, osteosarcoma is one of the most heterogeneous cancer types from a genetic point of view with, for instance, in a series of 44 samples more than 80 point mutations and 4 deletions related to 82 different genes [10]. Despite this heterogeneity, more than 80% of osteosarcomas evidence a signature characteristic of Breast Cancer (BRCA) genes 1/2-deficient tumors that open new therapeutic options by using PARP inhibitors [9].

Unfortunately, the genetic alterations described above while explaining its initiation are unable to sustain osteosarcoma development by themselves. Indeed, based on the “seed and soil’ theory of Paget, it is now well recognized that osteosarcoma, like other cancers, requires

an adequate local microenvironment for its development [11,12]. This microenvironment can provide all metabolites and factors for the control of proliferation, drug resistance, dissemination or/and dormancy of osteosarcoma cells [5,13-15]. Then, the pressure exerted by microenvironment may be responsible for a specific selection of osteosarcoma cells with predominant specificities such as a high quiescent or a proliferative profile.

Thus, a combination between genetic aberrations and an adequate microenvironment can summarize the full history of osteosarcoma. The present review will give a brief overview of the main components of the osteosarcoma microenvironment with a more specific focus on immune infiltrates, their role in the pathogenesis of osteosarcoma and their therapeutic implications.

Cellular heterogeneity of the osteosarcoma microenvironment: key role of immune infiltrates

Osteosarcomas are bone-forming tumors developing in a natural bone environment. Bone remodeling is controlled by two main cell types called osteoblasts and osteoclasts, which are responsible for bone formation and resorption, respectively [16,17]. The crosstalk between these cell types is tightly regulated by cytokines and growth factors. Among these mediators, the Receptor Activator NFκB Ligand (RANKL)/RANK/Osteoprotegerin (OPG) system has merged as a key molecular triad controlling the bone remodelling [18]. RANKL expressed by stromal/osteoblastic cells as a membranous and/or a secreted form binds to RANK, a transmembranous receptor expressed by myeloid osteoclast precursors. Their interaction leads to the induction of specific signal transduction pathways integrating NFκB transduction factor, which leads to the activation of osteoclastic genes (e.g. *NFATc1, cathepsin K, TRAP*) and then to the osteoclast differentiation and bone resorption. Osteoclastogenesis is possible

thanks to co-factors such as M-CSF that allows the proliferation and survival of osteoclast precursors. OPG acts a soluble decoy receptor produced by stromal/osteoblastic cells. It is able to bind to RANKL and to interrupt the RANKL binding to RANK [19]. OPG is then a strong inhibitor of osteoclast differentiation/activation and bone degradation. Whether RANKL is mandatory to osteoclastogenesis as revealed by the marked osteopetrotic phenotype of RANKL-deficient mice [20], M-CSF can be substituted by various other soluble mediators defining a M-CSF-dependent canonical and a M-CSF-independent non-canonical pathway of osteoclastogenesis [21,22]. However, the RANKL/RANK/OPG system is not restricted to the bone tissue and is also involved in the control of angiogenesis and immune system [19]. Indeed, RANKL up-regulates the property of dendritic cells to stimulate the proliferation and survival of naive T-lymphocytes [23]. Furthermore, the survival of dendritic cells is up-modulated in the presence of RANKL [24]. These observations led to the development of a concept in which bone and immune tissues were functionally interconnected and it is at the origin of the "osteoimmunology" [25,26]. The RANKL/RANK/OPG system is associated to the pathogenesis of osteosarcoma [27-29], directly through RANK on the osteosarcoma cell surface [30] and indirectly by regulating osteoclast activities [31] and the tumor vascularization [32]. Based on this observation, a fully humanized antibody able to block specifically RANKL binding to RANK and to inhibit the RANKL-induced signalling has been developed and called Denosumab [33]. Recently, a complete remission in a patient suffering from a progressive osteosarcoma and treated but a combination sorafnib and denosumab [34] and a phase II clinical trial has been initiated same year incluiding aone arm with denosumabd therapy after complete resection of all sites of metastatic disease (NCT02470091, « Denosumab in treating patients with recurrent or refractory osteosarcoma ») [6].

During bone resorption, many factors trapped in the extracellular matrix are released and may modulate the behavior of cancer cells and/or the quality of their microenvironment. The role of osteoclasts in the pathogenesis of osteosarcoma is still controversial and whether the number of osteoclasts seems to correlate with the aggressiveness of the disease [35], some authors described a protective role of osteoclast against the development of lung metastases [36]. The hypothesis is that osteoclasts may play a differential function in osteosarcoma according to the stage of the disease with a pro-tumoral function in the early stage and with the destruction/reorganization of bone niches in the later stage. The loss of osteoclast could then allow the cancer cell spreading [36]. The role of osteoclasts in immune response has been recently strengthened [37]. Ibáñez *et al.* demonstrated that osteoclasts should be considered as immune-modulators and that they can induce immunogenic $CD4^+$ T cell responses upon inflammation. In this context, according to the stage of the osteosarcoma development, associated local inflammation may impact the T-cell responses almost partly through osteoclasts [37]. Consequently, osteoclasts can be considered as important regulators of osteosarcoma growth through either their resorptive or their immune functions.

Bone and bone marrow environments are rich in mesenchymal stem cells that are closely located to osteosarcoma cells. Therefore, dialogs between these cells are possible [13]. In 2010, Perrot *et al.* reported a case of a late local recurrence of osteosarcoma occurring more than 10 years after the initial pathology and 18 months after a "lipofilling" procedure [38]. In complement of this observation, the co-injection of purified mesenchymal stem cells promoted the osteosarcoma development in a murine model, then underlying a functional interaction between osteosarcoma cells and mesenchymal stem cells. Knowledges on this dialog will be described in details in the following chapter. Bone marrow microenvironment is also mainly associated with the hematopoietic homeostasis and consequently is enriched in

immune cells. The part of immunity and associated inflammation in the control of osteosarcoma is not yet fully understood. However, Liu *et al.* analyzed a cohort of 162 osteosarcoma patients and found a correlation between immune markers (e.g. Glasgow prognostic score, neutrophil-lymphocyte ratio), the occurrence of metastases and the overall survival [39]. Similarly, lymphocyte/monocyte ratio appeared associated to the overall survival of patients strengthening the role of immune cells in osteosarcoma development [40]. Immune infiltrates composed by heterogenous cell populations such as myeloid and lymphoid cells, which play a role in innate and adaptive immunity, have been detected in osteosarcoma [41,42] (Figure 1, Figure 2). Whether myeloid (monocytes, macrophages, dendritic cells) and T-lymphocytes are the main populations represented, few B lymphocytes and rare mast cells have also been identified and may be related to tumor growth [41]. $CD20^+$ B lymphocytes cells showed a modest disseminated infiltrate [42], and mast cells ($CD117^+$, $tryptase^+$) are modestly detected in the tumor mass [41,42], and mainly observed at the bone-tumor interface where osteolysis was occurring [41]. Osteosarcoma cells maintain mast cell viability and activities [43], which could be a source of RANKL [44]. The involvement of mast cells in bone biology has been recently identified [45]. These authors demonstrated the crucial role of mast cells in the triggering of local/systemic inflammation and the recruitment of immune cells following bone injury. In this context, mast cells could control the osteoclastogenesis process and then appear as actors of tissue repair [45].

Mesenchymal stem cells control osteosarcoma development: potential immuno-regulation activities

Several studies have confirmed that mesenchymal stem cells promote the proliferation of osteosarcoma cells both *in vitro* and *in vivo* [38, 46-49]. Mesenchymal stem cells, the low differentiated precursor of various cell-types including osteoblast [16], in contrast to differentiated osteoblasts appear unable to communicate with osteosarcoma cells through the

establishment of functional gap junctions [50]. Whatever, several groups have demonstrated that extracellular vesicles represent an important way of communication between mesenchymal stem cells and osteosarcoma cells [51,52]. Under stress environment such as local acidosis associated to tumor growth and peritumoral osteolysis, mesenchymal stem cells could secrete extracellular vesicles carrying proteins, mRNA and microRNA modulating osteosarcoma cell proliferation and stemness [14,51,52]. Acquired stemness properties (e.g. ability to form sarcospheres, expression of stem-associated genes such as *Nanog* and *Oct4*) by osteosarcoma cells appeared mediated by the secretion of IL-6 by mesenchymal stem cells [52]. Interestingly, Torreggiani *et al.* demonstrated recently the spontaneous transfer of multidrug resistance (e.g. MDR- 1, Pgp) from isolated doxorubicin-resistant osteosarcoma cells to sensitive cells underlining the multiple functional activities of extracellular vesicles [53]. The dialog between osteosarcoma cells and mesenchymal stem cells is bilateral, and mesenchymal stem cells can be educated by tumor-secreted extracellular vesicles [54]. Osteosarcoma extracellular vesicle-educated mesenchymal stem cells contained IL-6 and TGF-β and were able to promote the tumor progression in a preclinical model. This tumor growth was blocked by administration of an anti-IL6 receptor antibody [54].

Activated mesenchymal stem cells can exhibit immunomodulatory properties [55-57]. In this context, IFN-γ synergizes with TNF-α and/or IL-1β for activating mesenchymal stem cells which become immunosuppressive and then can inhibit T cell proliferation [56]. Mesenchymal stem cells act similarly toward B and NK cells [55]. Overall, these data revealed a marked crosstalk between osteosarcoma cells, mesenchymal stem cells and immune cells in favor to the tumor development and metastatic process.

Osteosarcoma cells modulate the balance between pro- and anti-tumor macrophages

Macrophages are at the crossroad of bone homeostasis and immunity and have central functions in osteoimmunology. The role of macrophages in bone biology has been extensively exemplified [58]. These cells are involved in bone resorption acting as osteoclast precursors in presence of M-CSF and RANKL [59] and contributing also to bone formation [60]. Indeed, macrophages control osteogenesis from mesenchymal stem cells, mechanism dependent on Oncostatin M signaling. The role of macrophages present in the vicinity of tumor cells, named "Tumor-Associated Macrophages" (TAMs), has been extensively studied [61-63]. TAMs control the local immunity, angiogenesis and regulate tumor cell migration and invasion. In addition, TAMs participate in the seating of cancer cells at the metastatic site by modelling the permissiveness of the host-tissues. TAMs are composed by a large variety of sub-populations which have been classified initially in M1 and M2 subtypes according to their differentiation and activities. M1-polarized macrophages are classified as anti-tumor cells and M2-polarized macrophages as pro-tumor regulators [63,64]. Macrophages invade massively osteosarcoma tissues [41,42,65] (Figure 2) and establish an immune-tolerant environment during tumor growth [42,64]. Indeed, Buddingh *et al*. showed that osteosarcoma patients contained both M1 and M2 macrophages and that the total number of macrophages was associated with a good survival. However, the M2-phenotype was associated with a bad prognosis [65]. Recently, Dumars *et al.* showed that TAMs are associated with a better overall survival of osteosarcoma patients, and a dysregulation of the balance between M1- and M2-TAMs in favor of M1 cells was detected in non-metastatic patients [42]. Interestingly, Han et al. revealed that the M2-TAMs number was correlated with the frequency of suppressive $TIM3^{+}PD1^{+}$ T lymphocytes in osteosarcoma and the depletion of M2-TAMs led to an increase of T-lymphocytes proliferation and associated increase of pro-inflammatory cytokines [66]. Patients with aggressive paediatric bone sarcomas exhibited an

increased number of peripheral $CD14^{+}HLA\text{-}DR^{low/neg}$ immunosuppressive monocytes as well as an increase of Cytotoxic T-Lymphocyte Associated Protein 4 positive ($CTL4^{+}$) T cells and $CD14^{+}$ macrophage infiltrates [67]. Tim3/Gal9 interactions between T cells and myeloid cells could also result in an immunosuppressive response and repressed inflammation [68]. Overall, these studies point out that intra-tumor T-lymphocyte immunosuppression could be exacerbated by M2-TAMs specifically in metastatic patients (Figure 2). In preclinical models of osteosarcoma, M2-type macrophages were correlated to an increase of tumor growth, vascularization and metastatic dissemination [69]. Whether on the one hand the number of macrophages correlated with the number of blood vessels ($CD31^{+}$)[69] and on the other hand $CD163^{+}$ M2-TAMs with $CD146^{+}$ perivascular cells [42], the vascular density did not correlated with prognosis [70]. The prognostic relevance of the vascular density in osteosarcoma is controversial. However, in a recent study, Kunz *et al.* observed in cohort of 131 patients that the good responders to chemotherapy evidenced a low vascular density of their tumor mass with an improvement of survival as expected [71].

Based on these observations, therapeutic strategies targeting macrophages have been developed. Liposome-encapsulated muramyl tripeptide (L-MTP-PE, commercialized as Mifamurtide) is a synthetic analog of a component of bacterial cell wall which acts as a nonspecific immuno-modulator triggering macrophages and T-cell reaction by the release of soluble mediators [71,72]. The induction of M1-TAMs with anti-tumor activity induced by L-MTP-PE required IFN–γ [73]. While an initial clinical trial combined with chemotherapy was in favor of a therapeutic benefit for patients with osteosarcoma, its design led to a controversy and its therapeutic interest still remains highly questionable [6]. The potential clinical interest of Mifamurtide is always under investigation in clinical trials in association with conventional chemotherapy (Table 1). Targeting of M2-TAMs has been also envisaged in osteosarcoma by

the use of all-trans retinoic acid [74] or dihydroxycoumarins [75], that inhibit the differentiation of M2-subtypes.

Oncolytic adenoviruses which have been engineered to replicate in cancer cells and not in normal cells were used with success in a pre-clinical model of osteosarcoma [76]. Macrophages could be used as delivery platform of specific oncolytic virus as already demonstrated in prostate cancer [77].

Lymphocytes subpopulations in osteosarcoma and therapeutic interests

T-lymphocytes constitute the second most represented cell-type infiltrate in osteosarcoma tissues [41,42] (Figure 1). Recent estimation showed that Tumor-Infiltrating Lymphocytes (TILs) are detectable in 75% of osteosarcoma with a peak around 86% in metastases [78]. Interestingly, $CD8^+/FOXP3^+$-ratio in biopsies prior chemotherapy allows the identification of patients with prolonged survival confirming the results previously obtained in dogs [79,80]. The data revealed that patients with a $CD8^+/FOXP3^+$-ratio above 3.08 had an improved survival what strengthened the key function played by T-lymphocyte infiltrates in the pathogenesis of osteosarcoma [80]. The identification of tumor-specific lymphocytes has led to the development of immunotherapeutic approaches based on the recovery of an effective and sustained anti-tumor immune response in patients [81,82]. Then, it was proposed that TIL would represent a selected population of T-cells with a higher specific immunological reactivity against tumor cells than the non-infiltrating lymphocytes, despite of their anergy by the microenvironment (e.g. M2-TAMs, mesenchymal stem cells) or/and by cancer cells [78]. Based on this hypothesis, expanded T-lymphocytes loaded with tumor antigens were initially used in melanoma with a relative success in metastatic patients [81,82]. TIL were isolated and expanded with success in osteosarcoma. However obtained levels were

too low in regard to the requirement for immunotherapy [83,84]. In addition, while expanded TILs had higher cytotoxic properties against allogeneic and autologous tumor cells compared to autologous blood peripheral lymphocytes (Figure 1A), a low expression of immunomodulatory molecules on osteosarcoma cells or/and the secretion of suppressive mediators may prevent activation and expansion of these TILs [78]. This local immunosuppression has been extensively studied in the last five years. Ligands of B7 protein family are usually expressed by antigen presenting cells and modulate T-lymphocyte activities. This family includes three subgroups: i) B7-1, B7-2, and ICOS-L; ii) Programmed Death Ligand 1 (PD-L1) and 2 (PD-L2); iii) B7-H3, B7x, and HERV-H LTR-associating 2 (HHLA2) [85,86]. Osteosarcoma cells express several members of the B7 family to escape to the immune cells. Osteosarcoma cells express HHLA2 and PDL-1 proteins. HHLA2 levels were not associated with the total number of TILs detectable. However, these authors observed a positive correlation with the presence of $CD3^+$ T-lymphocytes, $CD20^+$ B-lymphocytes, $CD1a^+$ dendritic cells and $CD68^+$ macrophages [78]. HHLA2 expression was higher in metastatic specimens than in non-metastatic samples and was correlated with metastatic disease and poor survival rate [78]. HHLA2 could then contribute to the local immunosuppression in osteosarcoma. Similarly, osteosarcoma cells express B7-H3 which is correlated with the aggressiveness of the disease and metastatic capacity [85,86]. Program Death Ligand-1 (PDL-1) and PDL-2 were also detectable in all osteosarcoma cases [89-94]. The binding of PDL-1 and PDL-2 to PD-1 expressed by T-lymphocytes negatively regulates activating signal initiated by T-cell receptor following the presentation of tumor peptides associated with the major-histocompatibility system (Figure 1B). The main functional consequence is the inhibition of their cytotoxic properties contributing to the local immunosuppression and then tumor progression. In parallel, PD-1 was expressed in TIL [95] and was increased in peripheral $CD4^+$ and $CD8^+$ T-lymphocytes [96]. Based on these data, the

blockade of PD-1/PDL-1 interaction in osteosarcoma-bearing mice led to a decreased tumor burden and an improvement of overall survival [97]. The aberrant expression in osteosarcoma, the expression of PD-1 by TILs and the therapeutic benefit for blocking PD-1/PDL-1 interaction in preclinical models have justified the design of clinical trials. Three clinical trials using anti-PD1 antibody (Pembrolizumab and Nivolumab) are currently recruiting patients with advanced disease (Table 1).

γδT lymphocytes are key effectors of innate immunity by harboring TCR enabling the recognition of non-peptidic antigens such as microbial metabolites (e.g. pyrophosphomonoesters and alkylamines) or synthetic compounds such nitrogen-containing bisphosphonates [98]. Internalization of nitrogen-containing bisphosphonates by peripheral blood mononuclear cells leads to intracellular accumulation of isopentenyl pyrophosphate (IPP). IPP then increases Vγ9Vδ2 T-cell proliferation (the main subtype of γδT-cells) in a Vγ9Vδ2 T-cell receptor (TCR)-dependent manner and facilitates their expansion. Due to their high representation into the tumor microenvironment and their cytotoxic capacity, Vγ9Vδ2 T cells stimulated immunotherapeutic approaches in osteosarcoma [99-100]. Unfortunately, whether this therapeutic approach was promising, there are recent emerging evidences of γδ T cell subsets with immunosuppressive activities modulated by PDL-1/PD-1 system [103]. Numerous immunotherapies were assessed to modulate the tumor microenvironment and to reactivate a prolonged and efficient immune response [6]. Adoptive cell therapies based on TIL as described above and generating antigen-specific lymphocytes [103-105], dendritic cells [106-112], NK cells [113-118] were developed mostly *in vitro* and in pre-clinical model of osteosarcoma. Several clinical trials are currently in progress for evaluating their therapeutic efficacy in patients (Table 1).

Patient-derived orthotopic mouse models of osteosarcoma as support of immunotherapy development

Patient-derived xenograft models consist in the implantation of small tumor fragments from sarcoma patients into immunodeficient NOD-SCID mice. The main advantage of the PDX-models is the possibility of expanding the tumor tissues by retaining the original tumor architecture. The expansion of tumour tissue in PDX-models can occur for several more passages and still keep the original tumour cell/stromal architecture in place [119-121]. Several models of PDX of osteosarcoma has been described in the literature for various assessment including epigenomic, drug testing and therapeutic development [122-126]. These immunodeficient were very recently used for analysing the tumor-targeting amino-acid-auxotrophic strain *Salmonella typhimurium* A1-R [125,126]. *Salmonella typhimurium* A1-R exhibit a marked potential for infecting cancer cells and inducing cell death. More interestingly, bacteria induced the regression of cisplatin-resistant relapsed tumors [126]. Bacteria could then enhance the immune system and decrease its permissiveness to cancer cells. PDX models can be humanized and represent a new tool for the development of immunotherapy [126].

Conclusions

Osteosarcomas are rare bone malignant tumors which can occur in children and adolescents without a mature immune system. Unfortunately, the rate of therapeutic response was not improved since decades and the survival of osteosarcoma patients remains very low, specifically for metastatic patients. The crosstalk established between cancer cells and their microenvironment fuels the tumor growth by inducing a local immunosuppressive environment. Bone cells, mesenchymal stem cells, macrophages and cancer cells act in concert to reduce the proliferation of infiltrating T lymphocyte and to limit the immune

response. The future challenge in osteosarcoma will be to reprogram the microenvironment in order to enhance antitumor immune responses.

Acknowledgements: We thank Gillian Griffiths (University of Sheffield, UK) for the corrections of the manuscript.

Funding: This work was supported by the Bone Cancer Research Trust (UK, research project number 144681).

References

[1] WHO. WHO Classification of Tumours of Soft Tissue and Bone. 4th Ed, 281-295(International Agency for Research on Cancer, 2013).

[2] D. Heymann, F. Redini, Bone sarcomas: pathogenesis and new therapeutic approaches, IBMS BoneKEy 8 (2011) 402-414.

[3] Mori K, Rédini F, Gouin F, Cherrier B, Heymann, Osteosarcoma: current status of immunotherapy and future trends. Oncol. Rep. 15 (2006) 693-700.

[4] M. Kansara, M.W. Teng, M.J. Smyth, D.M. Thomas, Translational biology of osteosarcoma, Nat. Rev. Cancer. 14 (2014) 722-35.

[5] A. Alfranca, L. Martinez-Cruzado, J. Tornin, A. Abarrategi, T. Amaral, E. de Alava, P. Menendez, J. Garcia-Castro, R. Rodriguez. Bone microenvironment signals in osteosarcoma development. Cell Mol. Life Sci. 72(2015) 3097-113.

[6] M.F. Heymann, H.K. Brown, D. Heymann D, Drugs in early development for the treatment of osteosarcoma, Expert Opin. Investig. Drugs 25 (2016) 1265-1280

[7] A.B. Mohseny, P.C.Hogendoorn, Concise review: mesenchymal tumors: when stem cells go mad, Stem Cells 29 (2011) 397-403.

[8] A.J. Mutsaers, C.R. Walkley, Cells of origin in osteosarcoma: mesenchymal stem cells or osteoblast committed cells? Bone 62 (2014) 56-63.

[9] M. Kovac, C. Blattmann, S. Ribi, J. Smida, N.S. Mueller, F. Engert, F. Castro-Giner, J. Weischenfeldt, M. Kovacova, A. Krieg, D. Andreou, P.U. Tunn, H.R. Dürr, H. Rechl, K.D. Schaser, I. Melcher, S. Burdach, A. Kulozik, K. Specht, K. Heinimann, S. Fulda, S. Bielack, G. Jundt, I. Tomlinson, J.O. Korbel, M. Nathrath, D. Baumhoer, Exome sequencing of osteosarcoma reveals mutation signatures reminiscent of BRCA deficiency. Nat. Commun. 6 (2015) 8940.

[10] M. Bousquet, C. Noirot, F. Accadbled, J. Sales de Gauzy, M.P. Castex, P. Brousset, A. Gomez-Brouchet. Whole-exome sequencing in osteosarcoma reveals important heterogeneity of genetic alterations. Ann. Oncol. 27 (2016) 738-744.

[11] T. Borovski, E. De Sousa, F. Melo, L. Vermeulen, J.P. Medema, Cancer stem cell niche: the place to be. Cancer Res. 71 (2011) 634-639.

[12] I.J. Fidler, The pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited, Nat. Rev. Cancer 3 (2003) 453-458.

[13] M. Cortini, S. Avnet, N. Baldini, Mesenchymal stroma: Role in osteosarcoma progression. Cancer Lett. 405 (2017) 90-99.

[14] H.K. Brown, M. Tellez-Gabriel, D. Heymann, Cancer stem cells in osteosarcoma., Cancer Lett. 386 (2017)189-195.

[15] N McGranahan, C. Swanton, Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. Cell 168 (2017) 613-628.

[16] P. Katsimbri, The biology of normal bone remodelling, Eur J Cancer Care (Engl) in press.

[16] F. Deschaseaux, L. Sensébé, D. Heymann, Mechanisms of bone repair and regeneration, Trends Mol. Med. 15 (2009) 417-29.

[17] A.V. Rousselle, D. Heymann, Osteoclastic acidification pathways during bone resorption, Bone 30 (2002) 533-40.

[18] S. Theoleyre, Y. Wittrant, S.K. Tat, Y. Fortun, F. Redini, D. Heymann, The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodelling, Cytokine Growth Factor Rev. 15 (2004) 457-475.

[19] M. Baud'huin, F. Lamoureux, L. Duplomb, F. Rédini, D. Heymann, RANKL, RANK, osteoprotegerin: key partners of osteoimmunology and vascular diseases, Cell. Mol. Life Sci. 4 (2007) 2334-2350.

[20] D.L. Lacey, E. Timms, H.L. Tan, M.J. Kelley, C.R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, H. Hsu, J. Sullivan, N. Hawkins, E. Davy, C. Capparelli, A. Eli, Y.X. Qian, S. Kaufman, I. Sarosi, V. Shalhoub, G. Senaldi, J. Guo, J. Delaney, W.J. Boyle, Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation, Cell 93(1998):165-176.

[21] H.J. Knowles, N.A. Athanasou, Canonical and non-canonical pathways of osteoclast formation, Histol. Histopathol. 24 (2009) 337-346.

[22] M. Baud'huin, R. Renault, C. Charrier, A. Riet, A. Moreau, R. Brion, F. Gouin, L. Duplomb, D. Heymann, Interleukin-34 is expressed by giant cell tumours of bone and plays a key role in RANKL-induced osteoclastogenesis, J. Pathol. 221 (2010) 77-86.

[23] D.M. Anderson, E. Maraskovsky, W.L. Billingsley, W.C. Dougall, M.E. Tometsko, E.R. Roux, M.C. Teepe, R.F. DuBose, D. Cosman, L. Galibert, A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function, Nature 390 (1997) 175-179.

[24] B.R. Wong, R. Josien, S.Y. Lee, B. Sauter, H.L. Li, R.M. Steinman, Y. Choi, TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor, J. Exp. Med. 186 (1997) 2075-2080.

[25] M.C. Walsh, Y. Choi, Biology of the RANKL-RANK-OPG System in Immunity, Bone, and Beyond, Front. Immunol. 5 (2014) 511.

[26] H. Takayanagi, Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems, Nat. Rev. Immunol. 7 (2007) 292-304.

[27] N. Renema, B. Navet, M.F. Heymann, F. Lezot, D. Heymann, RANK-RANKL signalling in cancer. Biosci. Rep. 36 (2016) pii: e00366.

[28] Y. Chen, M.A. Di Grappa,S.D. Molyneux, T.D. McKee, P. Waterhouse, J.M. Penninger, R. Khokha, RANKL blockade prevents and treats aggressive osteosarcomas, Sci .Transl. Med. 7 (2015) 317ra197.

[29] E. Grimaud, L. Soubigou, S. Couillaud, P. Coipeau, A. Moreau, N. Passuti, F. Gouin, F. Redini, D. Heymann, Receptor activator of nuclear factor kappaB ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis, Am. J. Pathol. 163 (2003) 2021-2031.

[30] K. Mori, B. Le Goff, M. Berreur, A. Riet, A. Moreau, F. Blanchard, C. Chevalier, I. Guisle-Marsollier, J. Léger, J. Guicheux, M. Masson, F. Gouin, F. Rédini, D. Heymann, Human osteosarcoma cells express functional receptor activator of nuclear factor-kappa B., J. Pathol. 211 (2007) 555-562.

[31] J. Rousseau, V. Escriou, F. Lamoureux, R. Brion, J. Chesneau, S. Battaglia, J. Amiaud, D. Scherman, D. Heymann, F. Rédini, V. Trichet, Formulated siRNAs targeting Rankl prevent osteolysis and enhance chemotherapeutic response in osteosarcoma models, J. Bone Miner. Res. 26 (2011) 2452-2462.

[32] Z. Benslimane-Ahmim, J. Pereira, A. Lokajczyk, B. Dizier, I. Galy-Fauroux, A.M.Fischer, D. Heymann, C. Boisson-Vidal, Osteoprotegerin regulates cancer cell migration through SDF-1/CXCR4 axis and promotes tumour development by increasing neovascularization, Cancer Lett. 395 (2017) 11-19.

[33] B. Gobin, M. Baud'huin, B. Isidor, D. Heymann, MF Heymann. Monoclonal antibodies targeting RANKL in bone metastasis treatment. In Monoclonal antibodies in oncology, Ed. Fatih M. Uckum, eBook Future Medicine Ltd (2012) 42-53.

[34] R. Cathomas, C. Rothermundt, B. Bode, B. Fuchs, R. von Moos, M. Schwitter . RANK ligand blockade with denosumab in combination with sorafenib in chemorefractory osteosarcoma: a possible step forward? Oncology 88 (2015) 257–260.

[35] S. Avnet, A. Longhi, M. Salerno, J.M. Halleen, F. Perut, D. Granchi, S. Ferrari, F. Bertoni, A. Giunti, N. Baldini. Increased osteoclast activity is associated with aggressiveness of osteosarcoma, Int. J. Oncol. 33 (2008) 1231-1238

[36] L. Endo-Munoz, A. Evdokiou, N.A. Saunders, The role of osteoclasts and tumour-associated macrophages in osteosarcoma metastasis, Biochim. Biophys. Acta 1826 (2012) 434-442.

[37] L. Ibáñez, G. Abou-Ezzi, T. Ciucci, V. Amiot, N. Belaïd, D. Obino, A. Mansour, M. Rouleau, A. Wakkach, C. Blin-Wakkach, Inflammatory Osteoclasts Prime TNFα-Producing $CD4_+$ T Cells and Express CX_3 CR1, J. Bone Miner. Res. 31 (2016) 1899-1908

[38] P. Perrot, J. Rousseau, A.L. Bouffaut, F. Rédini, E. Cassagnau, F. Deschaseaux, M.F. Heymann, D. Heymann, F. Duteille, V. Trichet, F. Gouin, Safety concern between autologous fat graft, mesenchymal stem cell and osteosarcoma recurrence, PLoS One. 5 (2010) e10999.

[39] B. Liu, Y. Huang, Y. Sun, J. Zhang, Y. Yao, Z. Shen, D. Xiang, A. He, Prognostic value of inflammation-based scores in patients with osteosarcoma, Sci. Rep. 6 (2016) 39862.

[40] T. Liu, X.C. Fang, Z. Ding, Z.G. Sun, L.M. Sun, Y.L. Wang, Pre-operative lymphocyte-to-monocyte ratio as a predictor of overall survival in patients suffering from osteosarcoma, FEBS Open Bio. 5 (2015) 682-687.

[41] Y. Inagaki, E. Hookway, K.A. Williams, A.B. Hassan, U. Oppermann, Y. Tanaka, E. Soilleux, N.A. Athanasou, Clin. Sarcoma Res. 6 (2016) 13.

[42] C. Dumars, J.M. Ngyuen, A. Gaultier, R. Lanel, N. Corradini, F. Gouin, D. Heymann, M.F. Heymann, Dysregulation of macrophage polarization is associated with the metastatic process in osteosarcoma, Oncotarget 7 (2016) 78343-78354.

[43] F. Levi-Schaffer, Z. Bar-Shavit, Osteoblast-like cell line maintains in vitro rat peritoneal mast cell viability and functional activity, Immunology 69 (1990) 145-149.

[44] A.S. Ali, A.S. Lax, M. Liljeström, I. Paakkari, N. Ashammakhi, P.T. Kovanen, Y.T. Konttinen, Mast cells in atherosclerosis as a source of the cytokine RANKL, Clin. Chem. Lab. Med. 44 (2006) 672-674.

[45] J. Kroner, A. Kovtun, J. Kemmler, J.J. Messmann, G. Strauss, S. Seitz, T. Schinke, M. Amling, J. Kotrba, J. Dudeck, A. Dudeck, A. Ignatius, Mast cells are critical regulators of bone fracture-induced inflammation and osteoclast formation and activity, J. Bone Miner. Res., in press.

[46] M. Cortini, A. Massa, S. Avnet, G. Bonuccelli, N. Baldini, Tumor-associated mesenchymal stromal cells promote osteosarcoma stemness and migratory potential via IL-6 secretion, PLoS One 11 (2016) e0166500.

[47] Y. Wang, Y, Chu, B. Yue, X; Ma, G. Zhang, H. Xiang, Y. Liu, T. Wang, X. Wu, B. Chen, Adipose-derived mesenchymal stem cells promote osteosarcoma proliferation and metastasis by activating the STAT3 pathway, Oncotarget 8 (2017) 23803-23816.

[48] B. Tu, J. Zhu, S. Liu, L. Wang, Q. Fan, Y. Hao, C. Fan, T.T. Tang, Mesenchymal stem cells promote osteosarcoma cell survival and drug resistance through activation of STAT3, Oncotarget 7 (2016) 48296-48308.

[49] F.X. Yu, W.J. Hu, B. He, Y.H. Zheng, Q.Y. Zhang, L. Chen, Bone marrow mesenchymal stem cells promote osteosarcoma cell proliferation and invasion, World J. Surg. Oncol. 13 (2015) 52.

[50] M. Tellez-Gabriel, C. Charrier, B. Brounais-Le Royer, M. Mullard, H.K. Brown, F. Verrecchia, D. Heymann, Analysis of gap junctional intercellular communications using a dielectrophoresis-based microchip, Eur. J. Cell. Biol. 96 (2017) 110-118.

[51] K.C. Vallabhaneni, M.Y. Hassler, A. Abraham, J. Whitt, Y.Y. Mo, A. Atfi, R. Pochampally, Mesenchymal/stem stromal cells under stress increase osteosarcoma migration and apoptosis resistance via extracellular resistance via extracellular vesicle mediated communication, PLoS One. 11 (2016) e0166027.

[52] S. Avnet, G. Di Pompo, T. Chano, C. Errani, A. Ibrahim-Hashim, R.J. Gillies, D.M. Donati, N. Baldini, Cancer-associated mesenchymal stroma fosters the stemness of osteosarcoma cells in response to intratumoral acidosis via NF-κB activation, Int. J. Cancer 140 (2017) 1331-1345.

[53] E. Torreggiani, L. Roncuzzi, F. Perut, N. Zini, N. Baldini, Multimodal transfer of MDR by exosomes in human osteosarcoma, Int J Oncol 49 (2016) 189-96.

[54] S.R. Baglio, T. Lagerweij, M. Pérez-Lanzón, X.D. Ho, N. Léveillé, S.A. Melo, A.M. Cleton-Jansen, E.S. Jordanova, L. Roncuzzi, M. Greco, M.A.J. van Eijndhoven, G. Grisendi, M. Dominici, R. Bonafede, S.M. Lougheed, T.D. de Gruijl, N. Zini, S. Cervo, A. Steffan, V. Canzonieri, A. Martson, K. Maasalu, S. Köks, T. Wurdinger, N. Baldini, D.M. Pegtel, Blocking tumor- educated MSC paracrine activity halts osteosarcoma progression, Clin. Cancer Res. 23 (2017) 3721-3733.

[55] L. Zachar, D. Bačenková, J. Rosocha, Activation, homing, and role of the mesenchymal stem cells in the inflammatory environment, J. Inflamm. Res. 9 (2016) 231-240.

[56] G. Ren, L. Zhang, X. Zhao, G. Xu, Y. Zhang, A.I. Roberts, R.C. Zhao, Y. Shi, Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide, Cell Stem Cell 2 (2008) 141-150.

[57] C. Lo Sicco, D. Reverberi, C. Balbi, V. Ulivi, E. Principi, L. Pascucci, P. Becherini, M.C. Bosco, L. Varisio, C. Franzin, M. Pozzobon, R. Cancedda, R. Tasso, Mesenchymal stem cell-derived extracellular vesicles as mediators of anti-inflammatory effects: endorsement of macrophage polarization, Stem Cells Transl. Med. 6 (2017) 1018-1028.

[58] M.N. Michalski, L.K. McCauley, Macrophages and skeletal health, Pharmacol. Ther. 174 (2017) 3-54.

[59] M. Baud'Huin, C. Charrier, G. Bougras, R. Brion, F. Lezot, M. Padrines, D. Heymann, Proteoglycans and osteolysis. Methods Mol. Biol. 836 (2012) 323-337.

[60] P. Guihard, Y. Danger, B. Brounais, E. David, R. Brion, J. Delecrin, C.D. Richards, S. Chevalier, F. Rédini, D. Heymann, H. Gascan, F. Blanchard, Induction of osteogenesis in mesenchymal stem cells by activated monocytes/macrophages depends on oncostatin M signaling, Stem Cells 30 (2012) 762-772.

[61] S. Gordon, F.O. Martinez, Alternative activation of macrophages: mechanism and functions, Immunity 32 (2010) 593-604.

[62] C.E. Lewis, J.W. Pollard, Distinct role of macrophages in different tumor microenvironments, Cancer Res. 66 (2006) 605-612.

[63] R. Noy, J.W. Pollard, Tumor-associated macrophages: from mechanisms to therapy, Immunity 41 (2014) 49-61.

[64] B.Z. Qian, J.W. Pollard, Macrophage diversity enhances tumor progression and metastasis, Cell 141 (2010) 39-51.

[65] E.P. Buddingh, M.L. Kuijjer, R.A. Duim, H. Bürger, K. Agelopoulos, O. Myklebost, M. Serra, F. Mertens, P.C. Hogendoorn, A.C. Lankester, A.M. Cleton-Jansen, Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating agents, Clin. Cancer Res. 17 (2011) 2110-2119.

[66] Q. Han, H. Shi, F. Liu, $CD163^{+}$ M2-type tumor-associated macrophage support the suppression of tumor-infiltrating T cells in osteosarcoma, Int. Immunopharmacol. 34 (2016) 101-106.

[67] P. Hingorani, M.L. Maas, M.P. Gustafson, P. Dickman, R.H. Adams, M. Watanabe, F. Eshun, J. Williams, M.J. Seidel, A.B. Dietz, Increased $CTLA\text{-}4^{+}$T cells and an increased ratio of monocytes with loss of class II ($CD14^{+}$ $HLA\text{-}DR^{lo/neg}$) found in aggressive pediatric sarcoma patients, J. Immunother. Cancer 3 (2015) 35.

[68] X. Li, Y. Chen, X. Liu, J. Zhang, X. He, G. Teng, D. Yu, Tim3/Gal9 interactions between T cells and monocytes result in an immunosuppressive feedback loop that inhibits Th1 responses in osteosarcoma patients, Int. Immunopharmacol. 44 (2017)153-159.

[69] A.I. Ségaliny, A. Mohamadi, B. Dizier, A. Lokajczyk, R. Brion, R. Lanel, J. Amiaud, C. Charrier, C. Boisson-Vidal, D. Heymann, Interleukin-34 promotes tumor progression and metastatic process in osteosarcoma trhough induction of angiogenesis and macrophage recruitment, Int. J. Cancer 137 (2015) 73-85.

[70] P. Kunz, J. Fellenberg, L. Moskovszky, Z. Sápi, T. Krenacs, I. Machado, J. Poeschl, B. Lehner, M. Szendrõi, P. Ruef, M. Bohlmann, A.L. Bosch, V. Ewerbeck, R. Kinscherf, B. Fritzsching, Improved survival in osteosarcoma patients with atypical low vascularisation. Ann. Surg. Oncol. 22 (2015) 489-496.

[71] K. Mori, K. Ando, D. Heymann, Liposomal muramyl tripeptide phosphatidyl ethanolamine: a safe and effective agent against osteosarcoma pulmonary metastases, Expert Rev. Anticancer Ther. 8 (2008) 151-159.

[72] K. Ando, M. Mori, N. Corradini, F. Redini, D. Heymann, Mifamurtide for the treatment of nonmetastatic osteosarcoma, Expert Opin. Pharmacother. 12 (2011) 285-292.

[73] J.H. Pahl, K.M. Kwappenberg, E.M. Varypataki, S.J. Santos, M.L. Kuijjer, S. Mohamed, J.T. Wijnen, M.J. van Tol, A.M. Cleton-Jansen, R.M. Egeler, W. Jiskoot, A.C. Lankester, N.W. Schilham, Macrophages inhibit human osteosarcoma cell growth after activation with the bacterial cell wall derivative liposomal muramyl tripeptide in combination with interferon-γ, J. Exp. Clin. Cancer Res. 33 (2014) 27.

[74] Q. Zhou, M. Xian, S. Xiang, D. Xiang, X. Shao, J. Wang, J. Cao, X. Yang, B. Yang, M. Ying, Q. He, All-trans retinoic acid prevents osteosarcoma metastasis by inhibiting M2 polarization of tumor-associated macrophages, Cancer Immunol. Res. 5 (2017) 547-559.

[75] Y. Kimura, M. Sumiyoshi, Antitumor and antimetastatic actions of dihydroxycoumarins (esculetin or fraxetin) through the inhibition of M2 macrophage differentiation in tumor-

associated macrophages and/or G1 arrest in tumor cells, Eur. J. Pharmacol. 746 (2015) 115-125.

[76] M. Garcia-Moure, N. Martinez-Vélez, A. Patiño-García, M. M. Alonso, Oncolytic adnovirus as a therapeutic approach for osteosarcoma: a new hope, J. Bone Oncol., in press

[77] M. Muthana, S. Rodrigues, Y.Y. Chen, A. Welford, R. Hughes, S. Tazzyman, M. Essand, F. Morrow, C.E. Lewis, Macrophage delivery of an oncolytic virus abolishes tumor regrowth and metastasis after chemotherapy or irradiation, Cancer Res. 73 (2013) 490-495.

[78] P. Koirala, M.E. Roth, J. Gill, S. Piperdi, J.M. Chinai, D.S. Geller, B.H. Hoang, A. Park, M.A. Fremed, X. Zang, R. Gorlick, Immune infiltration and PD-L1 expression in the tumor microenvironment are prognostic in osteosarcoma, Sci. Rep. 6 (2016) 30093.

[79] B. Fritzsching, J. Fellenberg, L. Moskovszky, Z. Sápi, T. Krenacs, I. Machado, J. Poeschl, B. Lehner, M. Szendrõi, A.L. Bosch, L. Bernd, M. Csóka, G. Mechtersheimer, V. Ewerbeck, R. Kinscherf, P. Kunz, $CD8^+/FOXP3^+$ ration in osteosarcoma microenvironment separates survivors from non-survivors: a multicentre validated retrospective study, Oncoimmunology 4 (2015) e990800.

[80] B.J. Biller, A. Guth, J.H. Burton, S.W. Dow, Decreased ratio of CD8+ T cells to regulatory T cells associated with decreased survival in dogs with osteosarcoma, J. Vet. Intern. Med. 24 (2010) 1118-1123.

[81] M.H. Geukes Foppen, M. Donia, I.M. Svane, J.B. Haanen, Tumor-infiltrating lymphocytes for the treatment of metastatic cancer. Mol. Oncol. 9 (2015) 1918-1935.

[82] C.S. Hinrichs, S.A. Rosenberg, Exploiting the curative potential of adoptive T-cell therapy for cancer, Immunol. Rev. 257 (2014) 56-71.

[81] L. Rivoltini, F. Arienti, A. Orazi, G. Cefalo, M. Gasparini, C. Gambacorti-Passerini, F. Fossati-Bellani, G. Parmiani, Phenotypic and functional analysis of 3ymphocytes infiltrating paediatric tumours, with a characterization of the tumour phenotype, Cancer Immunol. Immunother. 34 (1992) 241-51.

[84] S. Théoleyre, K. Mori, B. Cherrier, N. Passuti, F. Gouin, F. Rédini, D. Heymann, Phenotypic and functional analysis of lymphocytes infiltrating osteolytic tumors: use as a possible therapeutic approach of osteosarcoma, BMC Cancer 5 (2005) 123.

[85] X. Zang, J.P. Allison, The B7 family and cancer therapy: costimulation and coinhibition, Clin. Cancer Res. 13 (2007) 5271-5279.

[86] F.A. Schildberg, S.R. Klein, G.J. Freeman, A.H. Sharpe, Coinhibitory pathways in the B7-CD28 ligand-receptor family, Immunity 44 (2016) 955-972.

[87] S.J. Yin, W.J. Wang, J.Y. Zhang, Expression of B7-H3 in cancer tissue during osteosarcoma progression in nude mice, Genet. Mol. Res. 14 (2015) 14253-61.

[88] L. Wang, Q. Zhang, W. Chen, B. Shan, Y. Ding, G. Zhang, N. Cao, L. Liu, Y. Zhang, B7-H3 is overexpressed in patients suffering osteosarcoma and associated with tumor aggressiveness and metastasis, PLoS One 8 (2013) e70689.

[89] N. Pinto, J.R. Park, E. Murphy, J. Yearley, T. McClanahan, L. Annamalai, D.S. Hawkins, E.R. Rudzinski, Patterns of PD-1, PD-L1, and PD-L2 expression in pediatric solid tumors, Pediatr. Blood Cancer in press.

[90] J.K. Shen, G.M. Cote, E. Choy, P. Yang, D. Harmon, J. Schwab, G.P. Nielsen, I. Chebib, S. Ferrone, X. Wang, Y. Wang, H. Mankin, F.J. Hornicek, Z. Duan, Programmed cell death ligand 1 expression in osteosarcoma, Cancer Immunol. Res. 2 (2014) 690-698.

[91] Y.T. Sundara, M. Kostine, A.H. Cleven, J.V. Bovée, M.W. Schilham, A.M. Cleton-Jansen, Increased PD-L1 and T-cell infiltration in the presence of HLA class I expression in metastatic high-grade osteosarcoma: a rationale for T-cell-based immunotherapy, Cancer Immunol. Immunother. 66 (2017) 119-128.

[92] A.E.M. van Erp, Y.M.H. Versleijen-Jonkers, M.H.S. Hillebrandt-Roeffen, L. van Houdt, M.A.J. Gorris, L.S. van Dam, T. Mentzel, M.E. Weidema, C.D. Savci-Heijink, I.M.E. Desar, H.H.M. Merks, M.M. van Noesel, J. Shipley, W.T.A. van der Graaf, U.E. Flucke, F.A.G. Meyer-Wentrup, Expression and clinical association of programmed cell death-1, programmed death-ligand-1 and CD8+ lymphocytes in primary sarcomas is subtype dependent, Oncotarget in press.

[93] Z. Zhu, Z. Jin, M. Zhang, Y. Tang, G. Yang, X. Yuan, J. Yao, D. Sun, Prognostic value of programmed death-ligand 1 in sarcoma: a meta-analysis, Oncotarget. 2017 Jul

[94] N. Maekawa, S. Konnai, T. Okagawa, A. Nishimori, R. Ikebuchi, Y. Izumi, S. Takagi, Y. Kagawa, C. Nakajima, Y. Suzuki, Y. Kato, S. Murata, K. Ohashi, Immunohistochemical Analysis of PD-L1 Expression in Canine Malignant Cancers and PD-1 Expression on Lymphocytes in Canine Oral Melanoma, PLoS One 11 (2016) e0157176.

[95] W. Zheng, H. Xiao, H. Liu, Y. Zhou, Expression of programmed death 1 is correlated with progression of osteosarcoma, APMIS 123 (2015) 102-107.

[96] D.M. Lussier, L. O'Neill, L.M. Nieves, M.S. McAfee, S.A. Holechek, A.W. Collins, P. Dickman P, J. Jacobsen, P. Hingorani, J.N. Blattman, Enhanced T-cell immunity to osteosarcoma through antibody blockade of PD-1/PD-L1 interactions, J. Immunother. 38 (2015) 96-106.

[97] Z. Li , Potential of human γδ T cells for immunotherapy of osteosarcoma, Mol. Biol. Rep. 40 (2013) 427-437.

[98] D. Heymann, B. Ory, F. Gouin, J.R. Green, F. Rédini F. Bisphosphonates: new therapeutic agents for the treatment of bone tumors. Trends Mol. Med. 10 (2004) 337-343.

[99] M. Liu, L.L. Sun, Y.J. Li, H.Y. Li, J. Zhang, B.H. Li, Z.M. Ye, Trastuzumab enhanced the cytotoxicity of Vγ9Vδ2 T cells against zoledronate-sensitized osteosarcoma cells, Int. Immunopharmacol. 28 (2015) 160-167.

[100] M. Muraro, O.M. Mereuta, F. Carraro, E. Madon, F. Fagioli, Osteosarcoma cell line growth inhibition by zoledronate-stimulated effector cells, Cell Immunol. 249 (2007) 63-72.

[101] Z. Li, H. Peng, Q. Xu, Z. Ye, Sensitization of human osteosarcoma cells to Vγ9Vδ2 T-cell-mediated cytotoxicity by zoledronate, J. Orthop. Res. 30 (2012) 824-830.

[102] Z. Li, Q. Xu, H. Peng, R. Cheng, Z. Sun, Z. Ye, IFN-γ enhances HOS and U2OS cell lines susceptibility to γδ T cell-mediated killing through the Fas/Fas ligand pathway, Int. Immunopharmacol. 11 (2011) 496-503.

[103] C. Fleming, S. Morrissey, Y. Cai, J. Yan, γδ T Cells: Unexpected Regulators of Cancer Development and Progression, Trends Cancer 3 (2017) 561-570.

[104] C. DeRenzo, S. Gottschalk, Genetically modified T-cell therapy for osteosarcoma, Adv. Exp. Med. Biol. 804 (2014) 323-340.

[105] N. Ahmed, V.S. Salsman, E. Yvon, C.U. Louis, L. Perlaky, W.S. Wels, M.K. Dishop, E.E. Kleinerman, M. Pule, C.M. Rooney, H.E. Heslop, S. Gottschalk, Immunotherapy for osteosarcoma: genetic modification of T cells overcomes low levels of tumor antigen expression, Mol. Ther. 17 (2009) 1779-1787.

[106] N. Rainusso, V.S. Brawley, A. Ghazi, M.J. Hicks, S. Gottschalk, J.M. Rosen, N. Ahmed, Immunotherapy targeting HER2 with genetically modified T cells eliminates tumor-initiating cells in osteosarcoma, Cancer Gene Ther. 19 (2012) 212-217.

[107] C. Chauvin, J.M. Philippeau, C. Hémont, F.X. Hubert, Y. Wittrant, F. Lamoureux, B. Trinité, D. Heymann, F. Rédini, R. Josien, Killer dendritic cells link innate and adaptive immunity against established osteosarcoma in rats, Cancer Res. 68 (2008) 9433-9440.

[108] Y.T. He, Q.M. Zhang, Q.C. Kou, B. Tang, In vitro generation of cytotoxic T lymphocyte response using dendritic cell immunotherapy in osteosarcoma, Oncol. Lett. 12(2016) 1101-1106.

[109] M. Kawano, K. Tanaka, I. Itonaga, T. Iwasaki, M. Miyazaki, S. Ikeda, H. Tsumura, Dendritic cells combined with anti-GITR antibody produce antitumor effects in osteosarcoma, Oncol. Rep. 34 (2015) 1995-2001.

[110] M. Kawano, I. Itonaga, T. Iwasaki, H. Tsuchiya, H. Tsumura, Anti-TGF-β antibody combined with dendritic cells produce antitumor effects in osteosarcoma, Clin. Orthop. Relat. Res. 470 (2012) 2288-2294.

[111] X. Fang, C. Jiang, Q. Xia, Effectiveness evaluation of dendritic cell immunotherapy for osteosarcoma on survival rate and in vitro immune response, Genet. Mol. Res. 14 (2015) 11763-11770.

[112] D.K. Krishnadas, S. Shusterman, F. Bai, L. Diller, J.E. Sullivan, A.C. Cheerva, R.E. George, K.G. Lucas, A phase I trial combining decitabine/dendritic cell vaccine targeting

MAGE-A1, MAGE-A3 and NY-ESO-1 for children with relapsed or therapy-refractory neuroblastoma and sarcoma, Cancer Immunol. Immunother. 64 (2015) 1251-1260.

[113] N. Tarek, D.A. Lee, Natural killer cells for osteosarcoma, Adv. Exp. Med. Biol. 804 (2014) 341-353.

[114] L. Fernández, J. Valentín, M. Zalacain, W. Leung, A. Patiño-García, A. Pérez-Martínez, Activated and expanded natural killer cells target osteosarcoma tumor initiating cells in an NKG2D-NKG2DL dependent manner, Cancer Lett. 368 (2015) 54-63.

[115] S. Kiany, N. Gordon, Aerosol delivery of interleukin-2 in combination with adoptive transfer of natural killer cells for the treatment of lung metastasis: methodology and effect, Methods Mol. Biol. 1441 (2016) 285-295

[116] E.P. Buddingh, M.W. Schilham, S.E. Ruslan, D. Berghuis, K. Szuhai, J. Suurmond, A.H. Taminiau, H. Gelderblom, R.M. Egeler, M. Serra, P.C. Hogendoorn, A.C. Lankester, Chemotherapy-resistant osteosarcoma is highly susceptible to IL-15-activated allogeneic and autologous NK cells, Cancer Immunol. Immunother. 60 (2011) 575-586.

[117] S.R. Guma, D.A. Lee, Y. Ling, N. Gordon, E.S. Kleinerman, Aerosol interleukin-2 induces natural killer cell proliferation in the lung and combination therapy improves the survival of mice with osteosarcoma lung metastasis, Pediatr. Blood Cancer 61 (2014) 1362-1368.

[118] S. Fallarini, T. Paoletti, N. Orsi Battaglini, G. Lombardi, Invariant NKT cells increase drug-induced osteosarcoma cell death, Br. J. Pharmacol. 167 (2012) 1533-1549.

[119] D. Siolas, G.J. Hannon. Patient-derived tumor xenografts: transforming clinical samples into mouse models. Cancer Res. 73 (2013):5315-5319.

[120] Hoffman RM. Patient-derived orthotopic xenografts: better mimic of metastasis than subcutaneous xenografts. Nat. Rev. Cancer 15 (2015) 451-452.

[121] R.M. Hoffman. Patient-Derived Mouse Models of Cancer. Ed. Molecular and Translational Medicine Series. Eds. W.B. Coleman, G.J. Tsongalis. (2017).

[122] P. Guilhamon, L.M. Butcher, N. Presneau, G.A. Wilson, A. Feber, D.S. Paul, M. Schütte, J. Haybaeck, U. Keilholz, J. Hoffman, M.T. Ross, A.M. Flanagan, S. Beck. Assessment of patient-derived tumour xenografts (PDXs) as a discovery tool for cancer epigenomics. Genome Med.6 (2014) 116.

[123] K. Igarashi, T. Murakami, K. Kawaguchi, T. Kiyuna, K. Miyake, Y. Zhang, S.D. Nelson, S.M. Dry, Y. Li, J. Yanagawa, T.A. Russell, A.S. Singh, H. Tsuchiya, I. Elliott, F.C. Eilber, R.M. Hoffman. A patient-derived orthotopic xenograft (PDOX) mouse model of a cisplatinum-resistant osteosarcoma lung metastasis that was sensitive to temozolomide and trabectedin: implications for precision oncology. Oncotarget 8 (2017) 62111-62119.

[124] C. Blattmann, M. Thiemann, A. Stenzinger, E.K. Roth, A. Dittmar, H. Witt, B. Lehner, E. Renker, M. Jugold, V. Eichwald, W. Weichert, P.E. Huber, A.E. Kulozik. Establishment of a patient-derived orthotopic osteosarcoma mouse model. J. Transl. Med. 13 (2015) 136.

[125] T. Murakami, K. Igarashi, K. Kawaguchi, T. Kiyuna, Y. Zhang, M. Zhao, Y. Hiroshima, S.D. Nelson, S.M. Dry, Y. Li, J. Yanagawa, T. Russell, N. Federman, A. Singh, I., Elliott, R. Mastuyama, T. Chishima, K. Tanaka, I. Endo, F.C. Eilber, R.M. Hoffman. Tumor-targeting *Salmonella typhimurium* A1-R regresses an osteosarcoma in a patient-derived xenograft model resistant to a molecular-targeting drug. Oncotarget 8 (2017) 8035-8042 .

[126] K. Igarashi, K. Kawaguchi, T. Murakami, T. Kiyuna, K. Miyake, S.D. Nelson, S.M. Dry, Y. Li, J. Yanagawa, T.A. Russell, A.S. Singh, N. Yamamoto, K. Hayashi, H. Kimura, S. Miwa, H. Tsuchiya, F.C. Eilber, R.M. Hoffman. Intra-arterial administration of tumor-targeting Salmonella typhimurium A1-R regresses a cisplatin-resistant relapsed osteosarcoma in a patient-derived orthotopic xenograft (PDOX) mouse model. Cell Cycle 16 (2017) 1164-1170.

[127] H. Jespersen, M.F. Lindberg, M. Donia, E.M.V. Söderberg, R. Andersen, U. Keller, L. Ny, I.M. Svane, L.M. Nilsson, J.A. Nilsson. Clinical responses to adoptive T-cell transfer can be modeled in an autologous immune-humanized mouse model. Nat. Commun. 8 (2017) 707.

Table 1: Ongoing clinical trials in osteosarcoma based on immunotherapy

Effector/target	Phase	Title	NCT Number
T lymphocytes	I	To evaluate the efficacy of NY-ESO-1-specific T Cell Receptor (TCR) affinity enhancing specific T cell in solid tumors	03159585
	I	CR-αβ+ and CD19+ depleted KIR/KIR ligand-mismatched haploidentical hematopoietic stem cell transplant and zoledronate for pediatric relapsed/refractory hematologic malignancies and high risk solid tumors	02508038
	I	A Phase I trial of the humanized anti-GD2 antibody In children And adolescents With neuroblastoma, osteosarcoma, Ewing sarcoma and melanoma	00743496
	I	Activated T cells armed with GD2 bispecific antibody in children and young adults with neuroblastoma and osteosarcoma	02173093
	I	A phase I trial of T cells expressing an anti-GD2 chimeric antigen receptor in children and young Adults with GD2+ solid tumours	02107963
	I	iC9-GD2-CAR-VZV-CTLs/refractory or metastatic GD2-positive sarcoma/VEGAS	01953900
	II	Humanized monoclonal Antibody 3F8 (Hu3F8) with granulocyte-macrophage colony stimulating factor (GM-CSF) in the treatment of recurrent osteosarcoma	02502786
	II	Dinutuximab in combination with sargramostim in treating patients with recurrent osteosarcoma	02484443
Natural killer cells	I/II	Pilot study of expanded, activated haploidentical natural killer cell infusions for sarcomas	02409576
	II	Phase 2 STIR trial: haploidentical transplant and donor natural killer cells for solid tumors (STIR)	02100891
Dendritic cells	I	A phase I trial of dendritic cell vaccination with and without inhibition of myeloid derived suppressor cells by gemcitabine pre-treatment for children and adults with sarcoma	01803152
Macrophage	II	A Eurosarc study of mifamurtide in advanced osteosarcoma (MEMOS)	02441309
	II/III	ABCB1/P-glycoprotein Expression as biologic stratification factor for patients with non metastatic osteosarcoma (ISG/OS-2)	01459484
Avelumab (anti-PD-L1)	II	A Phase II Trial of Avelumab, A Fully Human Antibody That Targets Cells Expressing PD-L1, in Patients With Recurrent or Progressive Osteosarcoma	03006848
Pembrolizumab (anti-PD1)	II	SARC028: A phase II study of the anti-PD1 antibody pembrolizumab (MK-3475) in patients with advanced sarcomas	02301039
	II	PROMO: A study of pembrolizumab in patients with relapsed or metastatic osteosarcoma not eligible for curative surgery	03013127
Nivolumab (anti-PD1)	I/II	Nivolumab with or without ipilimumab in treating younger patients with recurrent or refractory solid tumors or sarcomas	02304458
Nivolumab + Ipilimumab (anti-CTL4)	II	A Phase II Single Arm Study Assessing Efficacy & Safety of Nivolumab Plus Ipilimumab in Nonresectable/Metastatic Sarcoma and Endometrial Carcinoma Patients With Somatic Deficient MMR as a Selection Tool	02982486

rhGMCSF + furin bifunctional shRNA blocks furin protein	I	Phase I Trial of Bi-shRNAfurin and GMCSF Augmented Autologous Tumor Cell Vaccine for Advanced Cancer	01061840

Figure legends

Figure 1: T lymphocyte infiltration of osteosarcoma tissues. (A) Immunohistochemistry demonstrating the $CD3^+$ infiltrate of human osteosarcoma tissues. CD3 cells are mainly composed by CD8 T lymphocyte populations and a low number of $CD4^+$ cells. Immune local microenvironment of osteosarcoma cells is also composed by B lymphocytes (CD20) and a modest CD117 cell (mast cell) infiltrate. Osteosarcoma cells express PDL-1 which binds to PD-1 and/or B7/1 expressed at the cell surface of T lymphocytes. The binding of PDL-1 to PD-1 is associated with inhibiting downstream signaling which counterbalances the activation signal triggered by tumor-associated antigen presented by the major-histocompatibility system (MHC) to T cell receptor (TCR) and results in the reduction of T cell proliferation and an increase of their apoptosis. **(B)** Rat tumor infiltration lymphocytes (TIL) isolated and expanded from OSRGa Sprague Dawley osteosarcoma [79]. Expanded TILs are composed CD4 and CD8 cells which show a higher *in vitro* cytotoxic activity measured by chromium release. Similar infiltrate was observed in human samples [80]. PBL: Peripheral Blood Leukocytes; CD25: Interleukin-2 receptor alpha chain.

Figure 2: Osteosarcoma tissues are invaded by numerous macrophages controlling the behavior of cancer cells. The number of $CD68^+$ Tumor-Associated Macrophages (TAMs) in primary tumors prior chemotherapy is quantitatively similar in non-metastatic and metastatic patients. However, the number of $INOS^+$ M1-polarized macrophages are higher in non-metastatic than in metastatic patients leading to a dysregulation of the balance between $INOS^+$ M1 and $CD163^+$ M2 macrophage subtypes in favor to M1 cells. Consequently, this dysregulation leads to the establishment of a local tolerance, an increase of tumor vascularization facilitating the metastatic process.

Figure 1

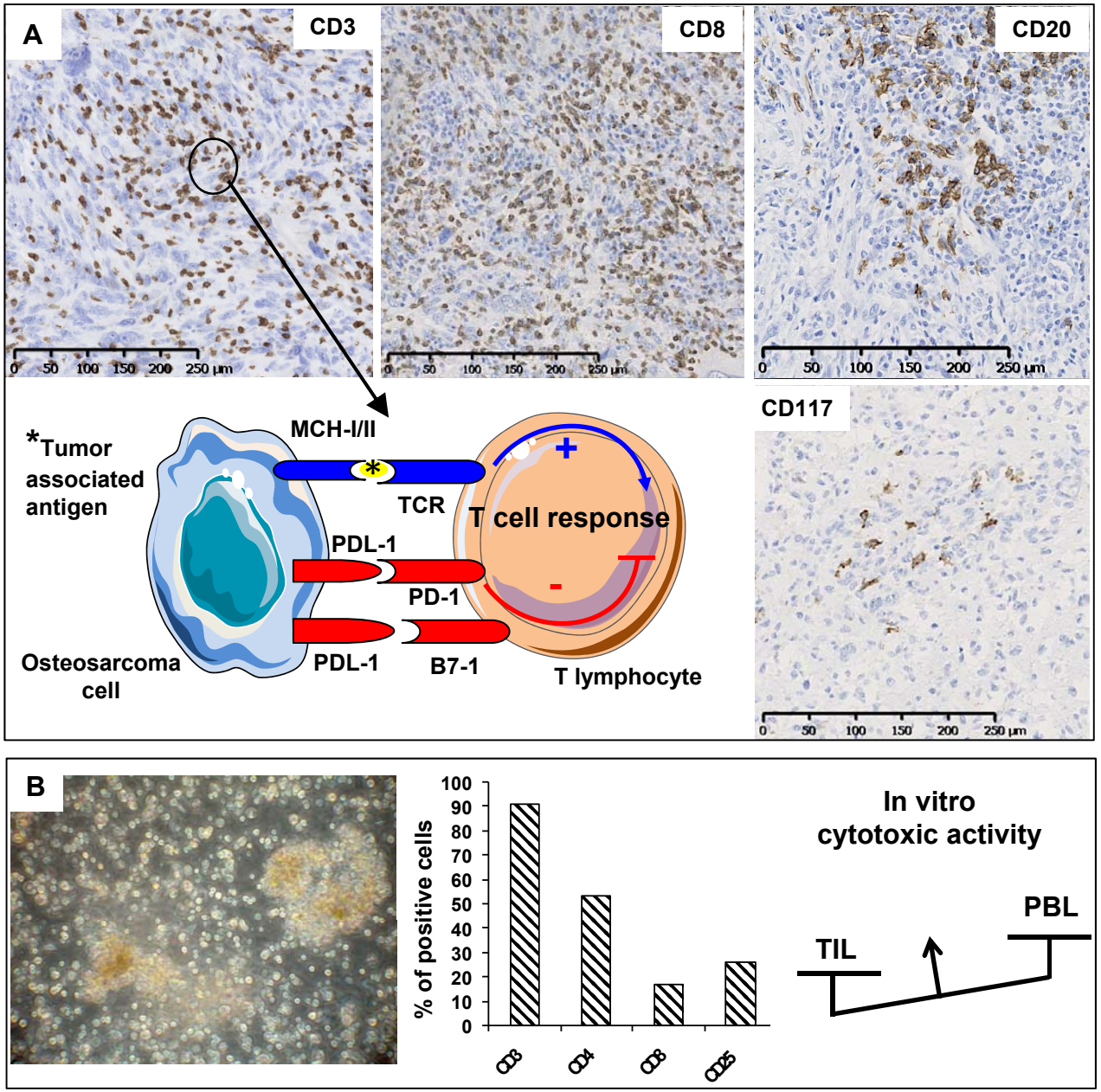
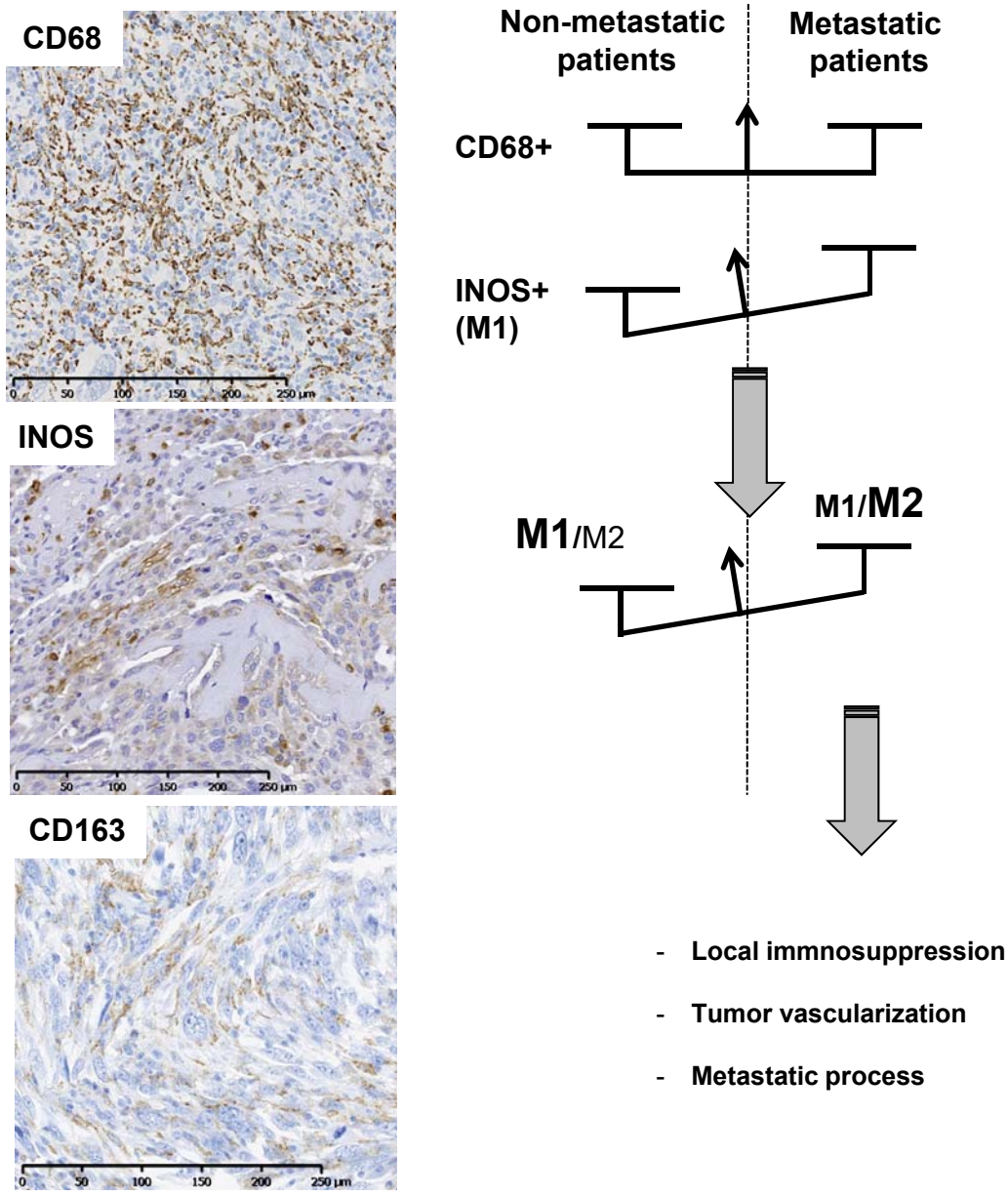

A
CD3
CD8
CD20
CD117
0 50 100 150 200 250 µm
*Tumor associated antigen
MCH-I/II
TCR
T cell response
+
-
PDL-1
PD-1
PDL-1
B7-1
Osteosarcoma cell
T lymphocyte
B
% of positive cells
100
90
80
70
60
50
40
30
20
10
0
CD3
CD4
CD8
CD25
In vitro cytotoxic activity
PBL
TIL

Figure 2


CD68
INOS
CD163
0 50 100 150 200 250 µm
Non-metastatic patients
Metastatic patients
CD68+
INOS+ (M1)
M1/M2
M1/M2
- Local immnosuppression
- Tumor vascularization
- Metastatic process