INNATE IMMUNITY IN OSTEOSARCOMA

The work presented in this thesis was financially supported by the Netherlands Organization for Health Research and Development (ZonMW grant 920-03-399) and EuroBoNet, a European Commission granted Network of Excellence for studying the pathology and genetics of bone tumors (grant number LSHC-CT-2006-018814).

Lay-out and printing: Off Page (www.offpage.nl)

Cover art: Trojan Horse, iStock photos.

ISBN: 978-94-6182-401-1

INNATE IMMUNITY IN OSTEOSARCOMA

Proefschrift

ter verkrijging van de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 5 maart 2014 klokke 13:45
door

Emilie Pauline Buddingh'
geboren te Ede
in 1979

PROMOTIECOMMISSIE:

Promotores:	Prof. Dr. R.M. Egeler
	Prof. Dr. P.C.W. Hogendoorn
Co-promotor:	Dr. A.C. Lankester
Leden:	Prof. Dr. A.H.M. Taminiau
	Prof. Dr. A.J. Gelderblom
	Prof. A.B. Hassan, University of Oxford, United Kingdom
	Dr. H. van den Berg, Emma Kinderziekenhuis, AMC, Amsterdam

'Contrariwise, if it was so, it might be;
and if it were so, it would be;
but as it isn't, it ain't.
That's logic.'

- Lewis Carroll, Through the Looking Glass.

TABLE OF CONTENTS

1.

Introduction

DEFINITION AND EPIDEMIOLOGY OF OSTEOSARCOMA

Osteosarcoma is a high-grade, intra-osseous malignancy in which the neoplastic cells produce bone (Fig. 1.1) [89]. It is the most common primary bone sarcoma. The World Health Organisation (WHO) has defined several major histological subtypes of osteosarcoma (Table 1.1) [217]. High-grade osteosarcoma occurs predominantly in children and young adults (Fig 1.2, incidence rate up to 8.4 per million in children aged 15 to 19 years), with a second peak of incidence in the elderly (incidence rate of 4.2 per million in the over 60 years age group) [177;248]. Incidence rates in adults aged 25 to 59 years are lowest at 1.7 per million. Males are affected more often than females at a ratio of 1.3 to 1.

PATHOGENESIS

Several lines of evidence suggest that osteosarcoma originates from mesenchymal stromal cells (MSCs) or early osteoblast precursor cells [181]. High-grade osteosarcoma commonly develops at an age and anatomical site of rapid proliferation and differentiation of MSCs, i.e. intramedullary near the growth plate of the long bones during or after the pubertal growth spurt. Long term *in vitro* expansion of murine MSCs results in oncogenic transformation of the cultured cells which form osteosarcoma-like tumors *in vivo* [113;116;179;183;259]. A patient transplanted with bone marrow (containing hematopoietic stem cells and MSCs) from a sibling was diagnosed with an osteosarcoma originating from donor cells 17 years later [19]. These data support the hypothesized mesenchymal stromal cell origin of osteosarcoma.

Most osteosarcomas arise sporadically, but some genetic or environmental factors increase the risk for developing osteosarcoma. Germ line mutations in the tumor suppressor genes *TP53* and *RB1* are associated with the Li-Fraumeni and hereditary retinoblastoma syndromes, respectively, both of which are associated with an increased risk to develop osteosarcoma and other types of cancer [231]. Mutations in the helicase genes *RECQL2*, *RECQL3* and *RECQL4* respectively cause Werner, Bloom and Rothmund-Thompson syndrome, all of which are associated with an increased risk of developing osteosarcoma [88;117;276]. In several other

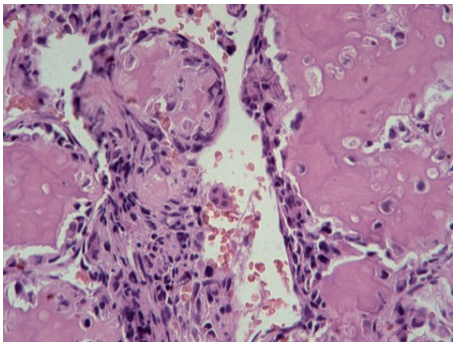


Fig. 1.1 Histology of conventional osteoblastic osteosarcoma. Hematoxylin and eosin stained section of diagnostic biopsy demonstrating osteoid production by neoplastic cells.

Table 1.1 Osteosarcoma classification according to the World Health Organisation [217]

Conventional osteosarcoma
- Osteoblastic (including sclerosing)
- Chondroblastic
- Fibroblastic
- Giant cell rich
- Osteoblastoma-like
- Epithelioid
- Clear cell
- Chondroblastoma-like
Telangiectatic osteosarcoma
Small cell osteosarcoma
Low grade central osteosarcoma
Parosteal osteosarcoma (low grade)
Periosteal osteosarcoma (intermediate grade)
High grade surface osteosarcoma

cancer syndromes an association with osteosarcoma has been reported, but is less well established. For example, according to Chowdry *et al.*, neurofibromatosis type 1 patients have a higher than expected incidence of bone sarcomas including osteosarcoma [43].

In a small percentage of osteosarcoma cases pre-malignant conditions can be identified, as has been reviewed in [105]. Well known precursor lesions are Paget's disease, fibrous dysplasia, dedifferentiated chondrosarcoma and giant cell tumor of bone [96;105;206]. Patients presenting with an osteosarcoma in the context of these conditions are often older than patients in whom no precursor lesion is identified.

Radiation exposure, for example in the treatment of other cancers, is the best known environmental risk factor in osteosarcoma, accounting for 0.5-5% of all new osteosarcoma cases [124;280].

Osteosarcoma is characterized by gross chromosomal instability with very complex polyploid karyotypes and marked cell-to-cell heterogeneity [231]. This is in contrast to many other sarcomas which can be defined by specific translocations, resulting in specific fusion transcripts, such as the *EWS-FLI1* transcript in Ewing sarcoma [230]. The highly complex chromosomal rearrangements as are present in osteosarcoma can occur as a result of a single catastrophic event, termed chromothripsis [72]. However, this probably has to occur in a susceptible background, either as a genetic predisposition or acquired as a *de novo* event. If chromothripsis occurs in the development of osteosarcoma and which somatic genetic or epigenetic aberration would confer susceptibility in osteosarcoma patients is as yet unknown. No specific chromosomal aberrations can be identified in osteosarcoma, but gain of chromosome 1 (present in 22% of examined karyotypes) and loss of chromosomes 9, 10, 13, and 17 (in 29%, 37%, 36% and 30% of specimens, respectively) occur in a significant proportion of osteosarcomas [30]. On

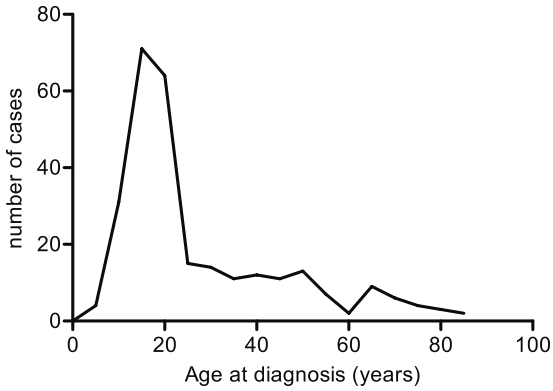


Fig. 1.2 Most osteosarcomas occur in children and young adults. Age distribution of 274 high-grade osteosarcoma patients diagnosed from 1990 to 2009 and treated at the Leiden University Medical Center. A second peak of incidence is often reported in the above sixty year age group, but is not apparent in our tertiary referral clinic, possibly reflecting referral bias.

a molecular genetic level, the TP53 and RB1 tumor suppressor pathways are often inactivated, resulting in loss of cell cycle control and unchecked cell proliferation [95;183;281].

DIAGNOSIS, TREATMENT AND PROGNOSIS

Most osteosarcomas are localized near the metaphyseal ends of the long bones, particularly in the distal femur, proximal tibia and proximal humerus. Patients often present with a history of pain and swelling of a few months duration. Symptoms sometimes seem to be precipitated by minor trauma. Rarely, patients present with a pathological fracture or functional impairment. Systemic symptoms are almost always absent. Radiographic examination usually reveals an osteolytic or osteosclerotic lesion with cortical involvement, a periosteal reaction and marked soft-tissue involvement (Fig. 1.3) [175]. Diagnostic biopsy of the lesion is required for a definitive diagnosis of osteosarcoma and for subtype classification (Fig. 1.1, Table 1.1). Staging studies include magnetic resonance imaging of the primary tumor to evaluate soft tissue expansion, computed tomography of the chest to evaluate the presence of pulmonary metastases and radionuclide bone scanning with technetium to evaluate the presence of bone metastases.

Prognosis for patients with osteosarcoma is mainly determined by the presence or absence of metastatic disease. About fifteen percent of patients have detectable metastatic disease at diagnosis and about 40 to 50 percent of initially non-metastatic patients subsequently develop detectable metastatic disease [172]. Overall survival for patients with metastatic disease is very poor at about twenty percent. Another major prognostic factor in osteosarcoma is the histological response to neo-adjuvant chemotherapy in the primary tumor, with less than ninety percent necrotic tumor area defined as prognostically unfavorable.

Before the introduction of chemotherapy in the management of osteosarcoma in the 1970s, long term survival rates were about 10% following complete resection of the tumor,

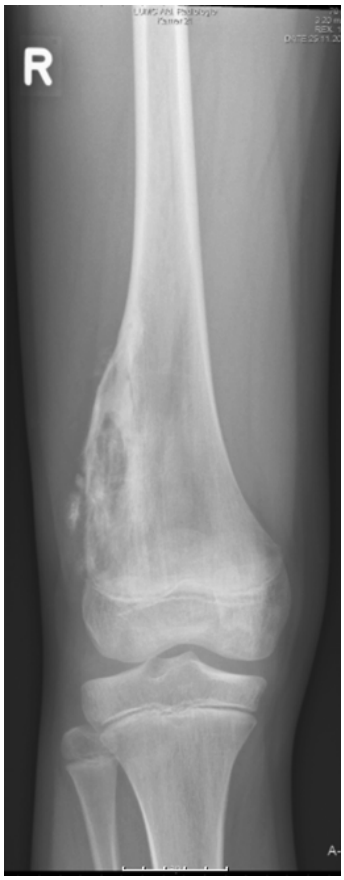


Fig. 1.3 Conventional radiograph demonstrating an osteosarcoma of the right femur in a 13 year old boy. Note the irregular structure of the bony matrix in metaphysis and diaphysis, the periostal reaction and the extension of the lesion into the soft tissue.

implicating that macroscopic or microscopic metastases are present in almost all patients at diagnosis. Since the introduction of chemotherapeutic treatment, overall long term survival of osteosarcoma has improved to about sixty percent. However, the overall survival rate has not improved substantially in the last twenty years, despite clinical trials investigating higher dosages and higher dose intensity chemotherapy regimens (Fig. 1.4). Currently, treatment consists of several rounds of neo-adjuvant and adjuvant chemotherapy in addition to radical surgical treatment of the primary tumor and metastases whenever feasible. Chemotherapeutic regimens have typically consisted of doxorubicin and cisplatin with or without high-dose methotrexate. Addition of etoposide and high-dose ifosfamide have been used in salvage regimens for non-resectable metastatic disease. A recent meta-analysis suggests that treatment with four or five drugs may not confer a survival advantage to treatment with a three drug regimen [8]. The addition of etoposide and ifosfamide for patients with poor histological response to the standard (neo-)adjuvant three drug regimen of methotrexate, adriamycin and doxorubicin is currently being evaluated in European and American Osteosarcoma Study Group (EURAMOS)-1 trial, for which results are due in 2015-2016 [160].

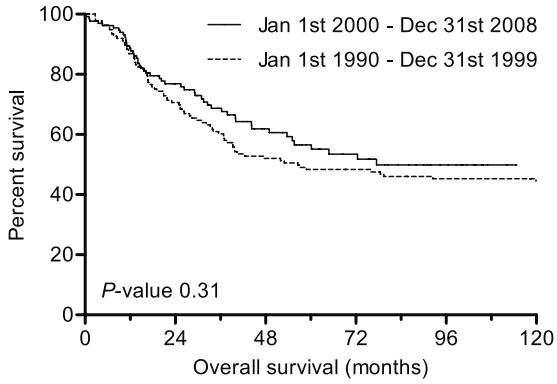


Fig. 1.4 Overall survival of osteosarcoma patients has not improved significantly since the 1990s (data of 274 high-grade osteosarcoma patients diagnosed from 1990 to 2008, treated at the Leiden University Medical Center)

INNATE IMMUNITY IN OSTEOSARCOMA PATIENTS

The innate immune system is the first-line defense system against pathogens and consists of physical barriers, cellular components and humoral components. Cellular components of the innate immune system such as granulocytes and macrophages are able to recognize and phagocytose pathogens through interaction of intracellular and surface membrane receptors with pathogen associated molecular patterns (PAMPs). Natural killer (NK) cells recognize and lyse virus infected cells when the balance of signals transduced via inhibitory and activating NK cell receptors is shifted towards activation [213]. There is increasing evidence that cells of the innate immune system such as macrophages and NK cells are able to not only detect and kill pathogens, but also tumor cells. In this thesis, we have studied the interaction between cells of the innate immune system -in particular macrophages and NK cells- and osteosarcoma cells, with the aim to provide preclinical data to guide future trials employing immunotherapeutic strategies.

Tumor-associated macrophages and cancer immunology

Infiltration of tumors with macrophages is often associated with worse prognosis. Several mechanisms have been proposed to explain the pro-tumorigenic effect of tumor-associated macrophages (TAMs). First, TAMs can express matrix-degrading proteins and thus facilitate tumor cell evasion and metastasis. Matrix metalloproteinase (MMP) expression by tumor stromal cells is associated with worse prognosis in many tumor types and MMP-9 expression by hematopoietic cells was essential for tumor progression in a murine squamous cell carcinoma model [50;63]. Second, TAMs can support angiogenesis through expression of specific cytokines and growth factors, for example vascular endothelial growth factor (VEGF), urokinase plasminogen activator and C-X-C chemokine receptor (CXCR)-2 ligands such as C-X-C chemokine ligand (CXCL)-8 [3;5;48;142]. Third, TAMs excrete immunosuppressive cytokines such as interleukin (IL)-10 and transforming growth factor- β (TGF-β), consequently hampering effective anti-tumor immunity [70;238]. These tumor promoting TAMs have many

characteristics in common with 'alternatively' IL-4 and IL-13 activated M2 type macrophages. M2 type macrophages express the scavenger receptor CD163 and have important tissue regenerative roles, for example in wound healing and angiogenesis. It is these homeostatic features that also promote growth and dissemination of tumor cells. Intratumoral infiltration with M2, or 'alternatively activated' macrophages is associated with increased angiogenesis, metastasis and decreased survival in many tumors, including sarcomas [78;138;266].

In contrast, 'classical activation' of macrophages by interferon-γ or microbial products results in expression of high levels of pro-inflammatory cytokines, such as IL-12, IL-1 and IL-6 [188]. These so-called M1 type macrophages have potent anti-tumor efficacy. They are able to kill tumor cells directly, through phagocytosis of tumor cells, reactive oxygen species and cytokine-induced cytotoxicity [109]. Additionally, they can recruit and activate NK and T cells and can initiate an adaptive immune cell response. In rare instances, high numbers of TAMs are associated with better survival of cancer patients [73;122]. It is presumed that the infiltrating macrophages in these tumors are polarized towards an M1 phenotype, but this has not been unequivocally proven.

M1 and M2 type activation of macrophages represent extremes of a spectrum (Fig 1.5). Macrophages demonstrate considerable phenotypic plasticity. Under the influence of environmental factors, polarization towards one or the other activation status can occur. To exploit this plasticity in an immunotherapeutic context, tumor resident M2 type macrophages may be classically activated towards an M1 phenotype by microbial cell products, or PAMPs. The successful utilization of immunostimulation in the treatment of sarcomas was first demonstrated by William B. Coley in 1891 [49;246]. Coley injected a mixture of streptococcal toxins into unresectable tumors and observed tumor regression in several sarcoma patients. More recently, post-operative infection has been shown to be associated with a better survival in osteosarcoma patients, possibly through activation of the innate immune system [114;136;242]. Treatment of osteosarcoma patients with the microbial cell wall product and macrophage activating agent muramyl tripeptide (MTP) in addition to standard chemotherapy improved overall survival in a recent clinical trial, although the exact mechanism remains unclear [170]. Another mechanism for classical activation of macrophages is through induction of immunogenic cell death. Often, chemotherapy induced cancer cell death is immunologically 'silent'. However, as a result of certain cytotoxic treatments, such as alkylating agents, oxaliplatin and ionizing radiation, immunogenic cell death can occur . The expression and cell surface translocation of damage-associated molecular patterns (DAMPs) such as calreticulin and high mobility group protein B1 trigger the activation of antigen presenting cells, similar to the response to PAMPs during infection [10;131;193].

NK cells

NK cells are innate immune cells that lack a clonally rearranged antigen-specific receptor. They have important regulatory functions through the expression of cytokines and chemokines and are potent effector cells. Cytolytic activity depends on the balance of between activating signals and inhibitory signals. High expression of ligands for the activating NK cell receptors Natural Killer Group 2, member D (NKG2D), DNAX accessory molecule-1 (DNAM-1) and the natural

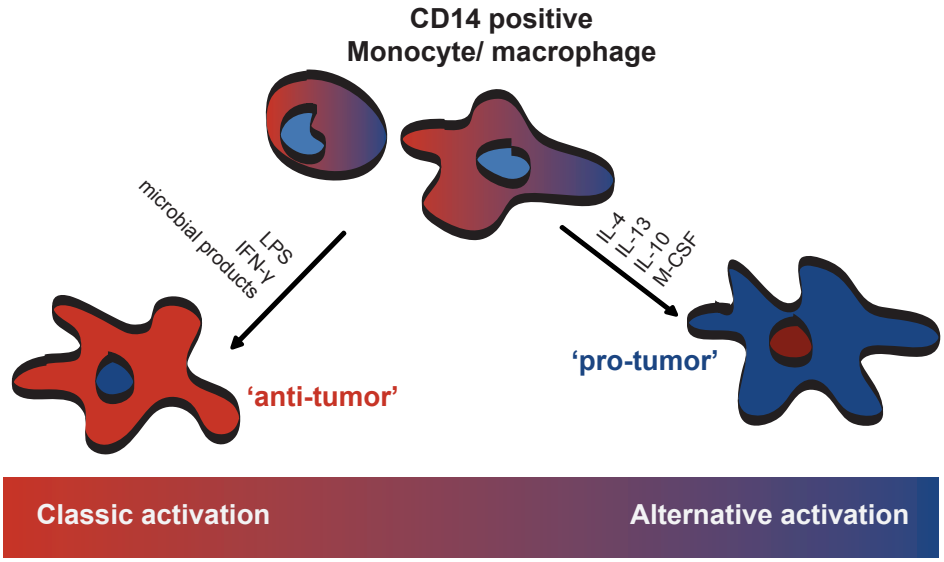


Fig. 1.5 M1 and M2 type macrophage activation represent extremes of a spectrum. Classically activated M1 type macrophages exhibit anti-tumor characteristics such as high expression of pro-inflammatory cytokines. Alternatively activated M2 type macrophages support tumor growth through tissue homeostatic features such as expression of matrix degrading proteins and support of angiogenesis. Adapted from [5] and [237].

cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 in conjunction with loss of expression of the ligands for the inhibitory NK cell receptors Killer Immunoglobulin-like Receptors (KIRs) and the C-type lectin heterodimer CD94/NKG2A on tumor cells can render them susceptible to NK cell mediated cytotoxicity (Fig. 1.6) [20;26;38;64;118;135;178;186;213;272].

Although NK cells have the natural ability to lyse tumor cells without the need for prior sensitization, NK cell mediated anti-tumor activity can be enhanced through the administration of NK cell activating cytokines such as IL-2 and IL-15, or the adoptive transfer of *ex vivo* activated NK cells [268;275]. There are several lines of evidence to suggest that treatment with NK cells or NK cell activating agents may be an effective adjunct to treatment of osteosarcoma patients. Previous pre-clinical studies have shown that osteosarcoma cell lines are sensitive to NK cell mediated cytolysis [104;132;143;156-159;165;191]. A murine model of post-operative osteomyelitis in osteosarcoma, in which anti-tumor activity was dependent on NK cells and monocytes, suggests that NK cell-mediated cytotoxicity also occurs *in vivo* [242]. A small cohort of osteosarcoma patients was treated with IL-2, which resulted in NK cell activation and a better outcome [151]. Interferon-α is an anti-proliferative and immunomodulatory cytokine,

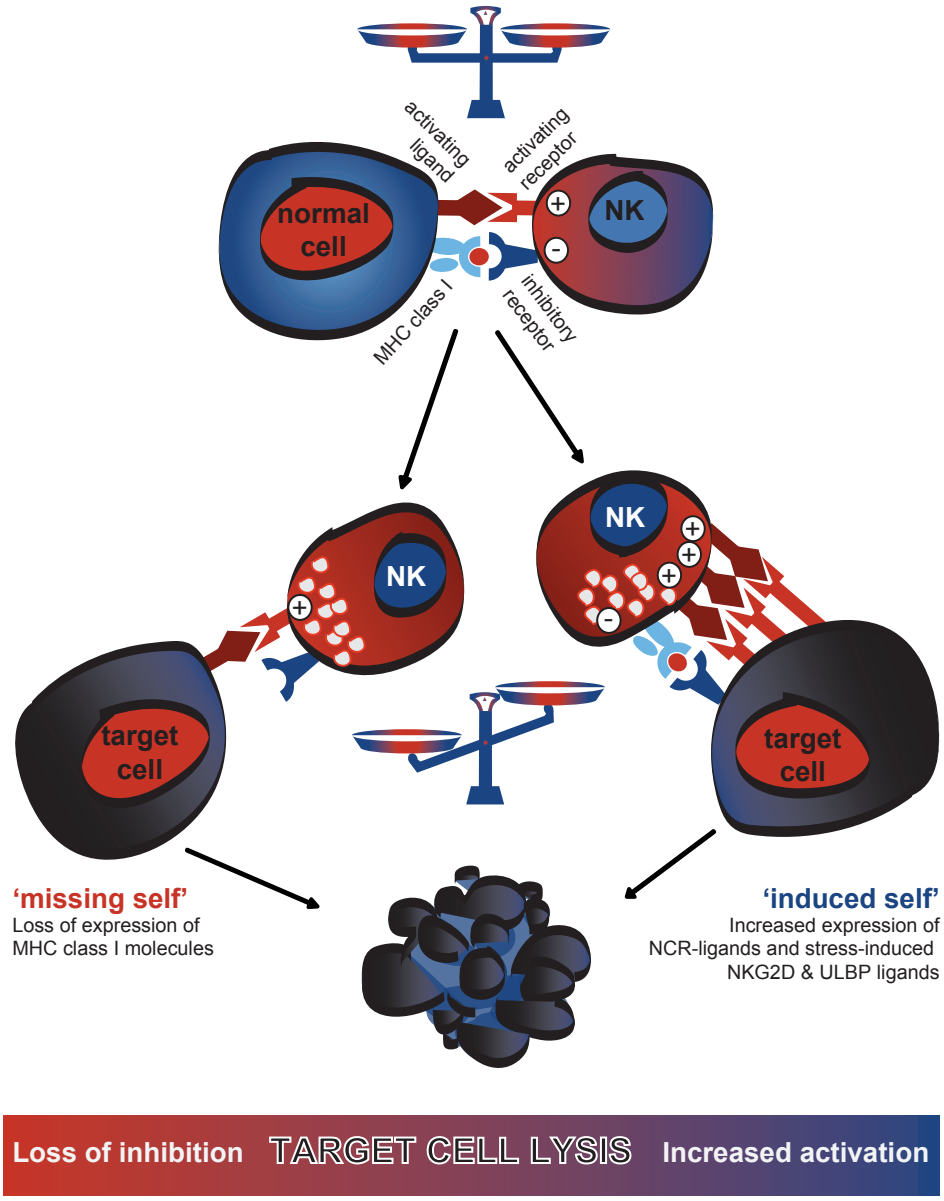


Fig. 1.6 NK cells engage in target cell lysis when the balance between inhibitory and activating signals is shifted towards activation. Adapted from [213].

administration of which results in activation of immune cell subsets such as monocytes and NK cells, in addition to direct anti-proliferative effects on tumor cells [31;62;154]. In Scandinavia, the addition of interferon to the treatment regimen of osteosarcoma patients resulted in a better outcome as compared to historical controls [249]. The efficacy of adjuvant interferon (IFN)-

α-2b is currently under study in the EURAMOS-1 trial. Preliminary data suggests no benefit, but follow-up of patients is ongoing [25;160;278].

AIMS AND OUTLINE OF THIS THESIS

A better understanding of the pathogenesis of high-grade osteosarcoma has the potential to identify novel targets for (immuno-)therapeutic interventions. MSCs are the proposed cell of origin of osteosarcoma. In **chapter 2**, the results of long term *in vitro* culture and genetic analyses of MSCs from osteosarcoma patients and healthy donors is described, to determine if MSCs from osteosarcoma patients are predisposed to malignant transformation. The main cause of death for osteosarcoma patients is pulmonary metastatic disease. In **chapter 3**, prognostic factors in pulmonary metastasized osteosarcoma patients are identified. This will aid in determining which patients are most likely to benefit from novel treatment modalities. In the next chapters, the constitutive *in vivo* interactions between the innate immune system and high-grade osteosarcoma are studied. Possible avenues for therapeutic exploitation of anti-osteosarcoma immunopotency are studied through *in vitro* manipulation of immune and target cells. In **chapter 4** the prognostic significance of intratumoral infiltration with macrophages is presented. In **chapter 5** cytotoxic activity of NK cells towards (chemotherapy resistant) osteosarcoma cells is studied. The recently closed EURAMOS-1 trial randomizes for treatment with interferon-α in patients with good histological response to neo-adjuvant chemotherapy [160]. In **chapter 6**, the molecular and functional effects of interferon treatment on the activation and anti-tumor activity of peripheral blood lymphocytes and monocytes of osteosarcoma patients, both *in vivo* and *ex vivo* is shown. In **chapter 7**, the findings of this thesis are summarized and discussed, and a view towards the direction of further studies is presented.

2.

Mesenchymal stromal cells derived from osteosarcoma patients do not transform during long-term culture

Emilie P. Buddingh, S. Eriaty N. Ruslan, Christianne M.A. Reijnders, Karoly Szuhai, Marieke L. Kuijjer, Helene Roelofs, Pancras C.W. Hogendoorn, R. Maarten Egeler, Anne-Marie Cleton-Jansen, Arjan C. Lankester

Submitted

ABSTRACT

In vitro expanded mesenchymal stromal cells (MSCs) are increasingly used as experimental cellular therapy. However, there have been concerns regarding the safety of their use, particularly with regard to possible oncogenic transformation. MSCs are the hypothesized precursor cells of high-grade osteosarcoma, a tumor with often complex karyotypes occurring mainly in adolescents and young adults. To determine if MSCs from osteosarcoma patients could be predisposed to malignant transformation we cultured MSCs of nine osteosarcoma patients and five healthy donors for an average of 649 days (range 601-679 days). Upon increasing passage, increasing frequencies of binucleate cells were detected, but no malignant transformation occurred in MSCs from either patients or donors. Also, we compared MSCs derived from osteosarcoma patients at diagnosis and from healthy donors using genome wide gene expression profiling. Hematopoietic cell specific Lyn substrate 1 (*HCLS-1*) was differentially expressed (fold change 0.25, *P*-value 0.0005) between MSCs of osteosarcoma patients (n=14) and healthy donors (n=9). In conclusion, this study shows that despite downregulation of *HCLS1* in MSCs of osteosarcoma patients and occurrence of binucleate cells in patient and donor derived MSCs, spontaneous transformation does not occur during long-term culture. In contrast to what has been reported for certain animal species, administration of *ex vivo* expanded human MSCs for therapeutic purposes is unlikely to result in sarcomas.

INTRODUCTION

Mesenchymal stromal cells (MSCs) are increasingly used as experimental cellular therapy in a wide range of conditions, such as graft-versus-host disease in the context of allogeneic bone marrow transplantation, auto-immune diseases and for regenerative purposes in for example myocardial injury or cartilage defects [15;46;137;198;264;274]. However, since MSCs have to be expanded *in vitro* to achieve sufficient numbers, there have been concerns regarding the safety of their use, particularly with regard to possible oncogenic transformation [219]. Cultured murine MSCs readily transform and form sarcoma-like tumors *in vivo* [113;116;179;183;259]. Similarly, MSCs derived from rhesus macaques become polyploid and subsequently aneuploid during long-term culture [107]. In contrast, human MSCs appear resistant to spontaneous *in vitro* transformation [22]. Studies reporting that human MSCs undergo malignant transformation *in vitro* have been retracted because of cross-contamination issues [56;218;221-223;260]. Despite the apparent difference between human and murine MSCs in their propensity to spontaneously transform *in vitro*, concerns remain. MSCs are hypothesized to be the precursor cells of high-grade osteosarcoma (OS) and a patient transplanted with bone marrow (containing hematopoietic stem cells and MSCs) from a sibling was diagnosed with OS originating from donor stem cells 17 years later [19]. This case demonstrates that donor-derived (pre-) cancerous MSCs can survive in a host and cause disease many years later. Another cause for concern is the observation that cultured MSCs can acquire chromosomal aberrations, although these do not seem to confer a selective growth advantage *in vitro* [18;254].

High-grade osteosarcoma is a malignant primary bone tumor which often occurs at a relatively young age [2]. OS tumor cells are characterized by aneuploid karyotypes and gross chromosomal instability [251]. Such highly complex chromosomal rearrangements can occur as a result of a single catastrophic event, termed chromothripsis [102;247]. However, this probably has to occur in a susceptible background, either as a genetic predisposition or acquired as a *de novo* event. In a murine model failed cytokinesis can lead to tetraploidy and subsequent tumorigenesis only in a p53 deficient host [77]. We previously showed loss of CDKN2A/p16 protein expression in tetraploid tumorigenic murine MSCs [183]. We hypothesized that normal MSCs from OS patients could be predisposed to malignant transformation, which might be identified by comparison of genome wide expression profiles of early passage MSCs from OS patients and healthy donors. Also, the presence of a pro-oncogenic predisposition could result in higher rates of spontaneous *in vitro* transformation during long-term culture. On the other hand, failure of these patient-derived cells to transform *in vitro* would be in line with previous reports in healthy individuals that spontaneous transformation of human MSCs *in vitro* is an extremely unlikely event. This would support the notion that therapeutic use of cultured (bone marrow-derived) MSCs is safe.

MATERIALS AND METHODS

Patients

Characteristics of OS patients and healthy stem cell donors can be found in Table 1. Bone marrow cells of OS patients were harvested under general anesthesia prior to start of the chemotherapeutic treatment. The site of MSC harvest (iliac crest) was different from the

location of the primary tumor (metaphyseal ends of the long bones) in all cases. Healthy donors were either identical sibling donors for patients with malignant or benign disease requiring hematopoietic stem cell transplantation; or haploidentical donors for either hematopoietical stem cell transplantation or the therapeutic infusion of MSCs for steroid-resistant graft-versus-host disease. Written informed consent was obtained from all patients and donors prior to bone marrow harvesting. The study was approved by the Institutional Review Board on Medical Ethics (LUMC Medical Ethics Committee (CME), P06.152).

Mesenchymal stromal cell cultures

Bone marrow derived mononuclear cells were obtained from 5 to 15 ml of heparinized bone marrow aspirate by density gradient centrifugation on Ficoll. Cells were plated on non-coated 75 cm2 polystyrene flasks at a cell density of 160000/cm2 in complete culture medium (LG-DMEM; Invitrogen, Paisley, United Kingdom) supplemented with penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (FBS; HyClone, Verviers, Belgium). We used a characterized and defined FBS batch preselected for its potential to support MSC expansion and continued to use this specific batch throughout the culture period. MSCs were plastic adherent and had spindle shaped morphology. Chondrogenic, adipogenic and osteoblastic differentiation were performed as described earlier [21]. Medium was refreshed twice a week and cells were replated when reaching 80-90% confluence at a density of 4000/cm2. The first nine OS patient and first five healthy donor MSC samples that were obtained were cultured long-term, the subsequent samples were used for confirmatory mRNA expression analysis (Table 1). Morphology of the cells was recorded and population doublings per passage (PD) were calculated using the log ratio of the harvesting cell count (N) to the starting (baseline) count (X0), divided by the log of 2 (PD = [log (N / X0)]/ log 2).

Flow cytometry

Expression of the cell surface markers CD73, CD90 and CD105 and absence of hematopoietic markers on MSCs was determined using flow cytometry, according to the statement by the International Society for Cellular Therapy [58]. Cells were detached using Trypsin/EDTA and washed in PBS/0,05% bovine serum albumin. Antibodies used were conjugated anti-CD86-fluorescein isothiocyanate (FITC) (cat. no. 555657), anti-HLA-DR-FITC (cat. no. 347400), anti-CD31-phycoerythrin (PE) (cat no 555446), anti-CD34-PE (cat no 348057), anti-CD73-PE (cat no 550257), anti-CD90-PE (cat no 555596), anti-CD3-peridinin chlorophyll protein(PerCP)-Cy5.5 (cat no 332771), anti-CD45-PerCPCy5.5 (cat no 332784), all from BD (San Diego, CA) and anti-CD105-PE (cat no SN6), from Ancell (Bayport, MN). Flow cytometry was performed on a FACScalibur, and analyzed using Cellquest software (both Becton Dickinson). Mean fluorescence intensity (MFI) ratio was calculated by determining the MFI of the specific staining relative to the MFI of the appropriate isotype control staining.

Analysis of binucleate cells

MSCs were grown on coverslips. Coverslips were washed with PBS and fixed in 4% PFA. Coverslips were incubated for ten minutes in 1µg/mL acridine orange (Sigma-Aldrich, Zwijndrecht, the Netherlands) to stain cytoplasm and 1µg/mL wheat-germ agglutinin with an Alexa Fluor® 594

Table 2.1 Characteristics of osteosarcoma patiens (OS) and healthy donors (HD) and overview of experiments. UI: unique identifier

Culture	OS(UI)/ HD	Sex	Age (y)	Histology	Location of the primary tumor	Included in experiment:		
						Microarray	qPCR	Long-term culture
001OS	OS(352)	M	12	Osteoblastic	Distal femur	y	y	y
002OS	OS(340)	F	13	Osteoblastic	Distal femur	y	y	y
003OS	OS(376)	F	13	Telangiectatic	Distal femur	y	y	y
004OS	OS(377)	F	14	Sclerosing	Proximal tibia	y	y	y
005OS	OS(348)	F	15	Telangiectatic	Distal tibia	n	n	y
006OS	OS(349)	M	8	Osteoblastic	Distal femur	y	y	y
007OS	OS(350)	F	15	Osteoblastic	Distal femur	n	n	y
008OS	OS(378)	F	9	Osteoblastic	Proximal humerus	y	y	y
009OS	OS(382)	M	15	Chondroblastic	Proximal humerus	y	y	y
010OS	OS(388)	M	14	Osteoblastic	Proximal humerus	n	y	n
011OS	OS(391)	F	14	Osteoblastic	Proximal tibia	n	y	n
012OS	OS(394)	F	5	Osteoblastic	Distal femur	n	y	n
013OS	OS(393)	F	14	Osteoblastic	Distal femur	n	y	n
014OS	OS(395)	M	10	Sclerosing	Distal femur	n	y	n
015OS	OS(396)	M	13	Osteoblastic	Distal femur	n	y	n
016OS	OS(402)	F	15	Sclerosing	Proximal tibia	n	y	n
HB	HD(HB)	M	15			y	y	y
HD3	HD(HD3)	F	27			y	y	y
HD5	HD(HD5)	M	50			y	y	y
MH	HD(MH)	M	15			y	y	y
TD1	HD(TD1)	F	11			y	y	y
TD2	HD(TD2)	F	43			n	y	n
TD3	HD(TD3)	F	43			n	y	n
TD4	HD(TD4)	F	25			n	y	n
TD5	HD(TD5)	M	5			n	y	n

conjugate (Invitrogen) in PBS to stain cell membranes. Following washes in PBS, coverslips were mounted using Vectashield with DAPI (Vector laboratories, Burlingame, CA) to stain nuclei. Images were acquired using a COHU 4910 series monochrome CCD camera (COHU, San Diego, CA) attached to a DM fluorescence microscope (Leica, Wetzlar, Germany) and analyzed using ImageJ software with the Cell Counter plug-in (National Institute of Mental Health, Bethesda, MD).

RNA isolation

RNA was isolated from frozen cell pellets of at least 1*10^6 undifferentiated MSCs at passage 2 to 5 using TRIzol reagent (Invitrogen). Cells were lysed in TRIzol, followed by phase separation in chloroform, precipitation using 2-propanolol and washing in 75% ethanol. RNA clean-up was

performed using the QIAGEN Rneasy mini kit (Venlo, the Netherlands) with on-column DNAse treatment. RNA quality and concentration were measured using an Agilent 2100 Bioanalyzer (Santa Clara, CA) and Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA), respectively.

cDNA synthesis, cRNA amplification, and Illumina Human v2.0 Expression BeadChip hybridization

Gene expression profiling was performed using Human-6 v2 Expression BeadChips (San Diego, CA) containing >48,000 transcript probes. Synthesis of cDNA, cRNA amplification, and hybridization of cRNA onto the Illumina Human v2.0 Expression BeadChips were performed as described previously [33;94].

qPCR

cDNA synthesis for qPCR was performed as described earlier [33]. qPCR was performed using the iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) according to the manufacter's instructions, reverse transcriptase negative (RT-) and H_2O controls were taken along for each sample. For normalization, three genes with stable expression in the microarray experiments were chosen (*CPSF6*, *GPR108* and *CAPNS1*). Data were normalized by geometric mean expression levels of the three reference genes using geNorm (http://medgen.ugent.be/jvdesomp/genorm/). Primer sequences can be found in Table S1. A standard curve was taken along for each primer set and used for quantification, PCR efficiencies ranged from 93 to 104%.

Karyotyping

Six early passage samples (three derived from OS patients, three derived from healthy donors) were subjected to a multicolor FISH based karyotyping test (COBRA-FISH) as described earlier [253]. From each sample at least twenty metaphase cells were recorded and analyzed.

Statistical analysis

Microarray data were normalized using the Cubic Spline normalization method with the Illumina BeadStudio Gene Expression Module. Microarray data are available at GEO using the accession no. GSE42572. Statistical analysis of microarray was performed using Significance Analysis for Microarrays, using a false discovery rate of 20% (SAM, http://www-stat.stanford.edu/~tibs/SAM/) [263]. Univariate statistical analyses were performed using GraphPad Prism software (version 5.01, La Jolla, CA). Two-sided *P*-values lower than 0.05 were determined to be significant.

RESULTS

MSCs of OS patients and healthy donors do not differ in expression of cell surface markers or differentiation capacity

We tested the MSC cultures from OS patients and healthy donors for phenotypic markers and functionality. Samples from 16/16 patients and 9/9 controls were able to differentiate into chondrogenic (Suppl. Fig. 2.1a), adipogenic (Suppl. Fig 2.1b and c) and osteoblastic lineages (Suppl. Fig. 2.1d and e). Also, all MSC cultures expressed CD73, CD90 and CD105 and lacked

expression of hematopoietic markers (Suppl. Fig. 2.1f). Level of expression of CD73, CD90 and CD105 as determined by MFI-ratios (specific staining/isotype control) did not differ between MSC cultures derived from OS patients and healthy donors (data not shown). Early passage samples from three OS patient derived MSCs and three healthy donor derived MSCs were karyotyped; no structural or numerical aberrations were observed in the analyzed samples (Suppl. Fig. 2.2): MSC001: 46,XY (26 metaphases), MSC003: 46,XX (27 metaphases), MSC008:46,XX (22 metaphases), MSC-HB: 46,XY (21 metaphases), MSC-MH: 46,XY (20 metaphases), MSC-TD: 46,XX (21 metaphases).

Long-term *in vitro* culture of MSCs results in increased binucleation but not in malignant transformation

MSCs of nine OS patients and five healthy donors were long term cultured (average number of days in culture 649, range 601-679 days). From each individual sample, duplicate cultures were established. There were no significant differences between growth rate, cumulative population doublings (median cumulative population doublings OS patients 34 *vs*. healthy donors 39; *P*-value Mann-Whitney U test 0.70)), passage number at termination of culture (mean passage number OS patients 21 *vs*. healthy donors 23; *P*-value Mann-Whitney U test 0.74) or time to growth arrest (median days to growth arrest OS patients 441 days *vs*. healthy donors 222 days; *P*-value Mann Whitney U test 0.15) between MSCs of OS patients and healthy donors. The cumulative population doublings are shown in Fig. 2.1 (averages of the duplicate cultures are shown per sample). All cultures exhibited rapid exponential growth in the first few passages. Later, proliferation slowed down and eventually stopped. At termination of the cultures, viable cells were present in all samples. Cultures were terminated at the end of the growth curves. At this point, none of the cultures had demonstrated an increase in proliferation indicative for spontaneous *in vitro* malignant transformation.

Morphology of the cells was inspected with every passage. Low passage cells were spindle-shaped, higher passage cells were larger. Upon increasing passage number, increasing frequencies of binucleate cells were noted using phase contrast microscopy. To quantify binucleation, cells were grown on coverslips and cytoplasm, cell membranes and nuclei were stained. Mono- and binucleate cells were counted (Fig. 2.2a-d). There was no difference in percentage of binucleate cells between MSCs derived from OS patients and healthy donors (Fig. 2.2e).

HCLS1 mRNA expression is downregulated in OS patient derived MSCs

Using microarray gene expression analysis, five genes were found to be differentially expressed between early passage healthy donor derived MSCs (n=5) and OS patient derived MSCs (n=7), with a false discovery rate of 20%. Expression of *HCLS1*, *ADM*, *EEF1A1*, *LOC644739* (or *WASF4*) and *LOC441155* was lower in patient derived MSCs as compared to healthy donor derived MSCs. To perform simultaneous technical and biological validation of the microarray results, the original low passage MSC samples were recultured and the original series expanded to now include four additional healthy donor derived MSCs (total of n=9) and seven additional OS patient derived MSCs (total of n=14). Four of the differentially expressed genes were validated using qPCR: hematopoietic cell specific Lyn substrate 1 (*HCLS1*), adrenomedullin

(*ADM*), eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*) and *LOC644739* (or WAS protein family, member 4; *WASF4*). *LOC441155* is a pseudogene for which we did not succeed in designing a sufficiently specific and efficient primer pair. In this second, newly cultured and expanded series, expression of *HCLS1* was significantly lower in OS patient derived MSCs as compared to healthy donor derived MSCs (Fig. 2.3, fold change 0.25, *P*-value 0.0005). The

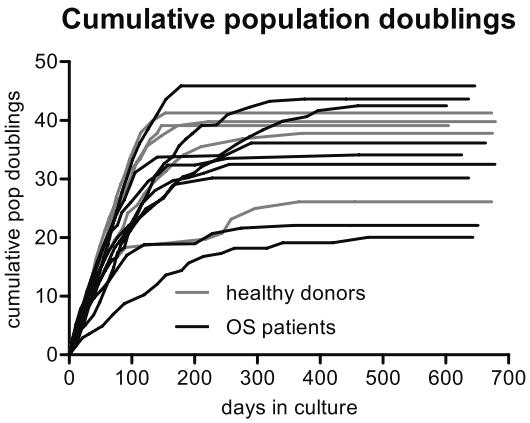


Fig. 2.1 Cumulative population doublings of cultured MSCs from nine osteosarcoma (OS) patients and five healthy donors. All samples were grown in duplicate, averages of each duplicate are shown.

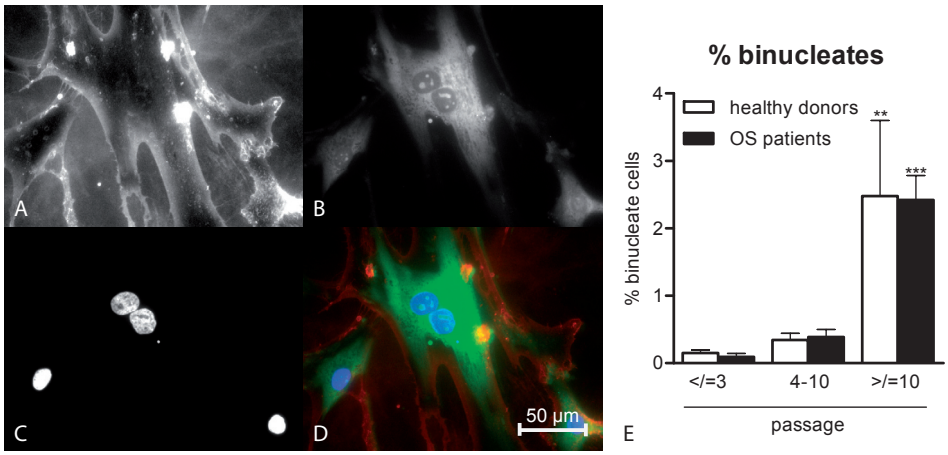


Fig. 2.2 Increased binucleation was noted upon increasing passage number. To facilitate cell staining and counting of nuclei, cells were grown on glass coverslips and stained using wheat-germ agglutinin (**a**, cell membranes), acridine orange (**b**, cytoplasm) and DAPI (**c**, nuclei), shown in 100x magnification. **d**, overlay with cell membrane in red, cytoplasm in green and nuclei in blue. A representative example of a high passage binucleate cell is shown. Note also the presence of a micronucleus (panel **c**). **e**, upon increasing passage number, more binucleate cells were noted, but no differences were seen between healthy donor-derived and osteosarcoma (OS) patient-derived MSCs (Kruskal-Wallis test *P*-value<0.0001, Dunn's post-test compared to early passage cells; P-value <0.01 noted as **; <0.001 = ***.

other genes were not differentially expressed in this series, perhaps because this was a newly expanded batch of cells. We tried to determine if the observed difference in *HCLS1* expression was also true at the protein level. Although HCLS1 protein could be clearly detected in the positive control cell line Jurkat, we were unable to reproducibly detect HCLS1 in MSCs (data not shown). For five OS patients we had mRNA available from both bone-marrow derived MSCs and the primary tumor. In these samples, expression of *HCLS1* was higher in tumor tissue as compared to MSCs, but there was no correlation between level of expression of *HCLS1* in low passage MSCs and in the corresponding tumor tissue (data not shown).

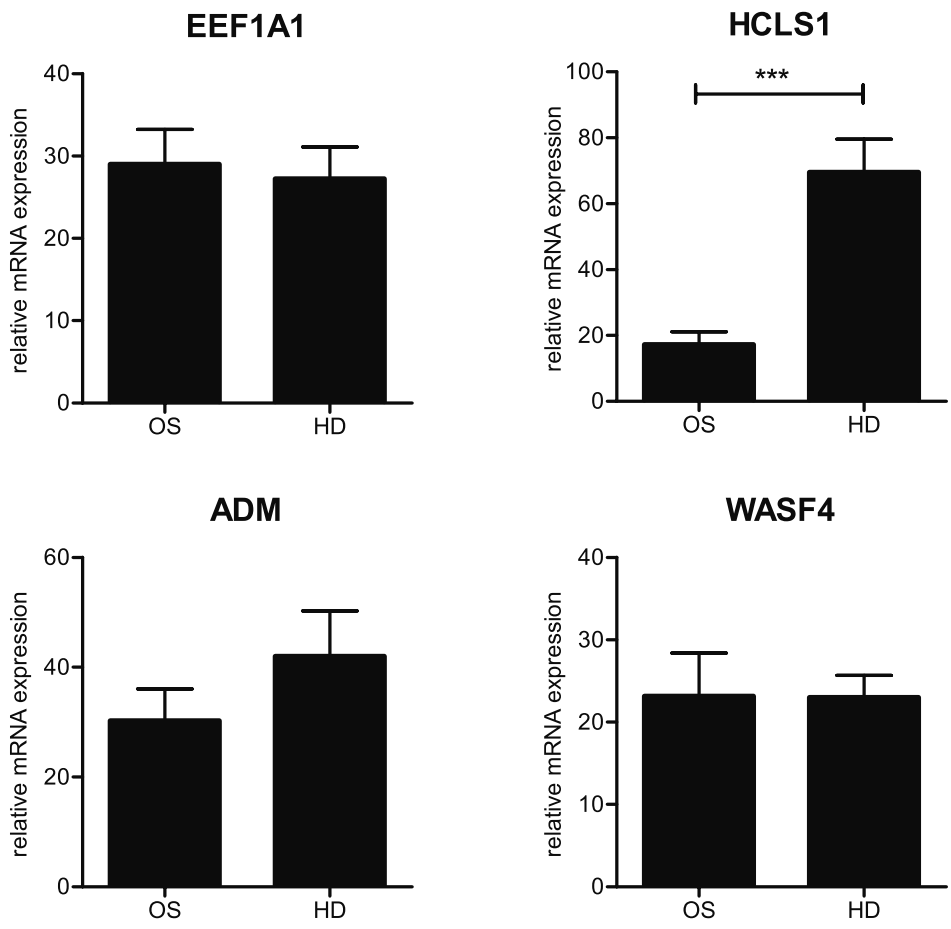


Fig. 2.3 Expression of *HCLS1* as determined by qPCR in bone-marrow derived MSCs no later than passage 5 is lower in MSCs derived from newly diagnosed osteosarcoma patients (OS; n=14) than in MSCs derived from healthy donors (HD; n=9). (***); *P*-value Mann Whitney-U test =0.0005. Expression levels for *EEF1A1, ADM* and *WASF4* were not significantly different.

DISCUSSION

In recent years, MSCs have gained increasing interest as a therapeutic modality for immune modulatory and regenerative purposes [46;137;198;264]. In order to achieve sufficient numbers of cells for therapeutic utility, MSCs need to be expanded *in vitro* prior to infusion. Since long-term culture of non-human MSCs can result in oncogenic transformation and the occurrence of sarcomas in the receiving hosts, there is ongoing debate regarding the safety of using cultured MSCs in clinical studies [209]. In the present study, we compared human MSCs derived from OS patients at diagnosis and from healthy donors to answer two questions. First, do human MSCs transform to a malignant phenotype when cultured *in vitro*, as has been shown for murine MSCs? Second, are MSCs derived from OS patients more likely to transform than healthy donor derived MSCs?

MSCs from patients and controls exhibited similar growth patterns during long-term *in vitro* culture, and we never observed malignant transformation or increased growth potential, even after almost two years of continuous culture. There were no phenotypical or functional differences between OS patient derived and healthy donor derived MSCs and early passage karyotypes were normal in both groups. Differentiation capacity and expression of specific cell surface markers was similar.

During prolonged culture, progressive shortening of telomeres occurs [123]. This can cause anaphase chromatin bridges, resulting in failed cytokinesis and consequently in binucleate cells [201]. In the context of loss of expression or function of tumor suppressor proteins and corresponding cell cycle checkpoints, tetraploidy and ultimately aneuploidy will occur [77]. Upon increasing passage, increasing frequencies of binucleate cells were noted; both in patient derived MSCs and healthy donor derived MSCs (Fig. 2.2). The lack of oncogenic transformation in the presence of increasingly high frequencies of binucleate cells, suggests that cell cycle checkpoints were functionally intact, both in MSCs derived from OS patients and from healthy donors.

In addition to the functional read-out of long-term culture, we also performed gene expression analysis on early passage cells. Expression of *HCLS1* was downregulated in MSCs from OS patients as compared to MSCs of healthy donors. *HCLS1* is primarily known for its role in the signaling cascade that follows B cell receptor activation. It is highly expressed in B-cell derived malignancies and is associated with a poor outcome [75;233]. Further studies are needed to determine if *HCLS1* might have a tumor suppressor function and if its loss of expression in OS patients derived MSCs has any relationship to *in vivo* tumorigenesis. Although we show no functional difference between healthy donor and OS patient derived MSCs, we do not advocate the use of MSCs from known cancer patients for clinical purposes. The relevance of the differential expression of *HCLS1* remains unknown and there might be other undetected (pre-) malignant alterations.

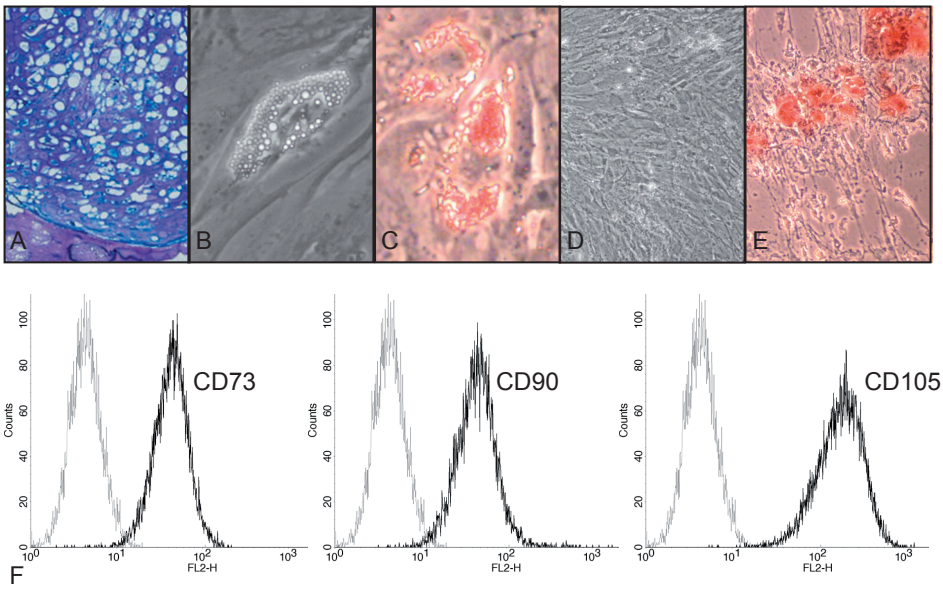
Both on a transcriptional and on a functional level, OS patient and healthy donor derived MSCs were very similar. There are several possible explanations for the observed similarities between OS patient derived and donor derived MSCs. First, we obtained MSCs from the iliac crest, while all tumors in our series were located at the metaphyseal ends of the long bones. OS occurs at a time and place of active growth and perhaps this pro-proliferative microenvironment (with high expression of growth factors) is an essential prerequisite for oncogenic transformation of MSCs.

Second, (pre-)oncogenic alterations may be present in only one or a few local mesenchymal tumor precursor cells and not in MSCs at a distant site such as the iliac crest. This could be due to somatic mosaicism, similar to what has been shown for the enchondromatosis syndromes Ollier disease and Maffucci syndrome [202]. Third, according to the 'multiple hit hypothesis', there may not have been sufficient 'hits' for *in vitro* transformation to occur, even if relevant predisposing alterations are present. Perhaps one or two additional 'hits' (for example loss of cell cycle checkpoint control) would be enough for oncogenic transformation to occur. Fourth, pre-malignant alterations may have been present in only a few cells, which would not lead to large enough differences in gene expression to be picked up by genome wide expression analysis. Finally, although there is compelling evidence to suggest MSCs are the precursor cells for human OS, this has not yet been unequivocally proven.

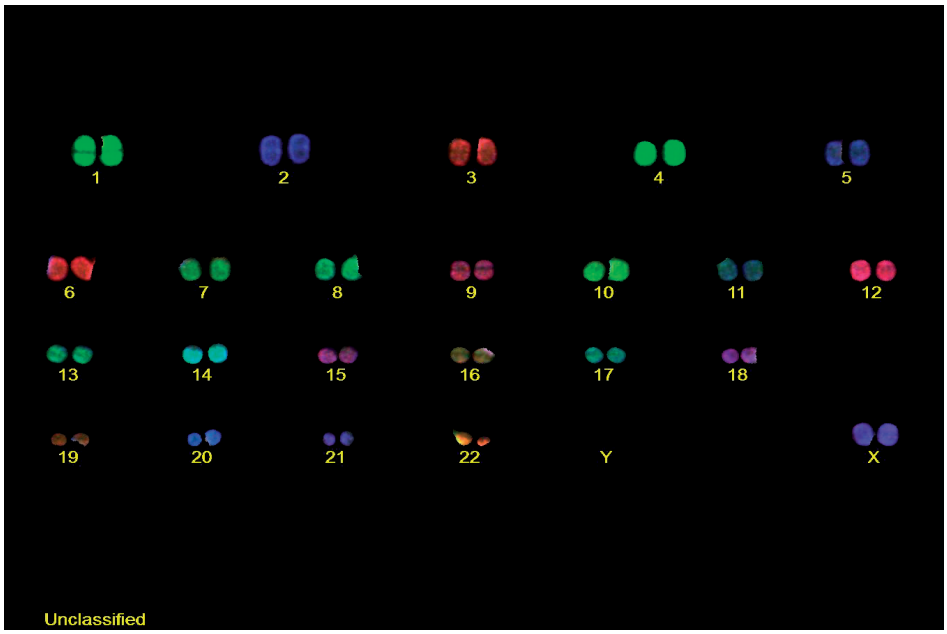
In conclusion, during long-term *in vitro* culture of human OS patient and healthy donor derived MSCs, oncogenic transformation did not occur. We could not confirm our hypothesis that MSCs of OS patients might have a higher propensity to oncogenic transformation than healthy donor derived MSCs. In contrast to what has been reported for other species, under the tested conditions, human MSCs do not easily transform. Therefore, administration of cultured human MSCs for therapeutic purposes is unlikely to result in sarcomas in the host.

ACKNOWLEDGMENT

The authors like to thank Ronald Duim for technical assistance, Marco Schilham for critical review, and Maarten Morsink for help in analysis of microarray data. Also, the authors would like to thank the medical and nursing staff of the departments of pediatrics and clinical oncology for help in obtaining informed consent.



Suppl. Fig. 2.1 Differentiation capacity and expression of membranous markers of MSCs. Representative examples are shown (40x magnification). **a**, chondrogenic differentiation was performed using cell pellet cultures and assessed using toluidine blue staining. Adipogenic differentiation was assessed using phase contrast microscopy (**b**) and Oil-red-O staining (**c**). Osteogenic differentiation was assessed using phase contrast microscopy (**d**) and Alizarin red staining (**e**). **f**, all samples expressed CD73, CD90 and CD105 (bold histograms) as determined by flow cytometry (isotype controle staining shown for comparison).



Suppl. Fig. 2.2 Representative example of karyotypic analysis of early passage MSCs (OS patient derived MSC003OS; 46,XX).

3.

Prognostic factors in pulmonary metastasized high-grade osteosarcoma

Emilie P. Buddingh, Jakob K. Anninga, Michel I.M. Versteegh, Antonie H.M. Taminiau, R. Maarten Egeler, Catherina S.P. van Rijswijk, Pancras C.W. Hogendoorn, Arjan C. Lankester, Hans Gelderblom

Pediatr Blood Cancer. 2010 Feb;54(2):216-21.

ABSTRACT

INTRODUCTION: Resection of pulmonary metastases has previously been reported to improve outcome in high-grade osteosarcoma (OS) patients. Factors influencing survival in OS patients with pulmonary metastases are important for clinical decision making. METHODS: All 88 OS patients with pulmonary metastases either at diagnosis or during follow-up treated at the Leiden University Medical Center between January 1, 1990 and January 1, 2008 under the age of 40 were included in this study, including 79 cases of conventional, eight cases of telangiectatic and one case of small cell OS. RESULTS: In total, 56 of 88 patients with pulmonary metastases were treated by metastasectomy. Resectability of pulmonary metastases was the main prognostic factor. In patients with primary non-metastatic OS, a longer relapse free interval to pulmonary metastases was significantly associated with better survival (P=0.02). Independent risk factors determining worse survival after metastasectomy in multivariate analysis were male sex (P=0.05), higher number of pulmonary nodules (P=0.03), and non-necrotic metastases (P=0.04). Whether surgery for recurrent pulmonary metastases was performed did not influence survival. Histological subtype of the primary tumor, histological response in the primary tumor after neoadjuvant chemotherapy, occurrence of local relapse, local resection or amputation of the primary tumor and age at diagnosis did not influence outcome. CONCLUSION: This cohort of patients with detailed follow-up data enabled us to identify important risk factors determining survival in OS patients with pulmonary metastases. We demonstrate that after repeated metastasectomies, a subset of patients can be cured.

INTRODUCTION

High-grade osteosarcoma (OS) is a malignant bone tumor mainly affecting adolescents and young adults [71]. Since the introduction of (neo-)adjuvant chemotherapy, long-term overall survival has improved to about 60%, with failure of therapy mainly attributed to chemoresistant metastatic disease. At diagnosis 15–25% of patients present with clinically detectable metastatic disease (synchronous pulmonary metastases) and about 40–50% of patients with primary non-metastatic disease experience relapse, mainly to the lungs (metachronous pulmonary metastases) [4;13;24;141;170;177;243].

Resection of pulmonary metastases with or without second-line chemotherapy has been reported to improve outcome in OS patients with pulmonary metastases and surgery is currently standard treatment in many institutions for patients in whom metastases are deemed resectable. Despite aggressive surgery, however, many patients still relapse. The two largest single-institution studies to date investigating prognostic factors determining survival of OS patients with lung metastases undergoing metastasectomy have had conflicting results. Studies conducted at the Rizzoli Institute concluded that higher numbers of pulmonary nodules, bilateral disease, and incomplete resection were independent prognostic factors for poor survival after metastasectomy [12;14;29]. This was confirmed in a large multi-center study and in several smaller studies [121;228;256;277]. In contrast, several studies concluded that neither number of pulmonary nodules nor other clinical parameters such as disease-free interval, bilateral disease or resection margins significantly affect survival [42;53;97]. In the subgroup of patients with metachronous disease, longer disease-free interval has been associated with a favorable outcome, both in smaller single-center studies and in large multi-center studies [83;121;256;277]. The role of second-line chemotherapy is unclear, with only some authors describing a moderate survival benefit when administered in addition to metastasectomy [23].

In the current study, we sought to determine factors determining outcome in a cohort of patients with extensive follow-up data with high-grade OS metastasized to the lungs, including 79 cases of conventional OS, eight cases of telangiectatic OS and one case of small cell OS.

PATIENTS AND METHODS

Definition of cohort

Between January 1990 and January 2008 197 patients under the age of 40 were treated for high-grade OS at the Leiden University Medical Center. Excluded were patients with insufficient follow-up data (n=12) and unresectable primary tumor (n=11). Of the remaining 174 patients, all 88 patients who had pulmonary metastases either at diagnosis or during follow-up were included in this study (Table 3.1). Patients were treated according to one of the consecutive European Osteosarcoma Intergroup (EOI) trials 80861 [243] and 80931 [141] (doxorubicin and cisplatinum, with or without high-dose methotrexate, bleomycin, cyclophosphamide, actinomycin- D, and vincristin) or according to the Euramos-1 trial [1] (doxorubicin, cisplatinum, and high-dose methotrexate with or without interferon-alpha or etoposide and ifosfamide).

Imaging studies

Pulmonary metastases were diagnosed by routine computed tomography (CT) scans of the lungs and additional staging with ^{99m}Tc-bone scintigraphy, and if needed magnetic resonance imaging (MRI). Follow-up included clinical investigations and X-rays of the primary site and lungs. Number and distribution of pulmonary nodules were noted in case of suspected pulmonary metastases.

Histopathology

Primary tumors were histologically classified according to the criteria of the World Health Organization [71]. Resected specimens of primary tumors were evaluated for histological response to neoadjuvant chemotherapy; good response was defined as less than 10% vital tumor tissue. Resected specimens of pulmonary metastases were evaluated for number of resected nodules and viability of resected tumor tissue. In addition, completeness of resection and occurrence of pleural contamination were noted on macroscopic and microscopic examination.

Statistical analysis

Unpaired t-tests, contingency analyses (χ^2 method) and univariate survival analyses (Kaplan–Meier method, log rank test for comparison of survival curves) were performed using GraphPad Prism 5.0. Multivariate survival analyses and survival analyses with continuous input variables were carried out according to the Cox proportional hazards model in SPSS version 16.0. Survival time was calculated from date of diagnosis to date of last follow-up or death (noted as overall survival) or from date of first metastatic event to date of last follow-up or death (noted as overall survival since metastasis). Two-sided *P*-values lower than 0.05 were determined to be significant; *P*-values between 0.05 and 0.10 were defined to be a trend.

RESULTS

Twenty-six of 88 patients had pulmonary metastases at diagnosis (synchronous pulmonary metastases, group A in Table 3.1) and 62 patients developed pulmonary metastases during follow-up (metachronous pulmonary metastases, group B in Table 3.1). The proportion of high-grade OS patients with clinically detectable pulmonary metastases did not change during the study period. About 15% of all high-grade OS patients had pulmonary metastases at diagnosis and 35% developed pulmonary metastases during follow-up. There was a male predominance in our cohort (71.6% males vs. 28.4% females) and there was a trend for males to have a worse overall survival (*P* logrank = 0.08). Most tumors were located in the distal femur (43/88, 48.9%), proximal tibia or fibula (18/88, 20.5%), and proximal humerus (8/88, 9.1%). Less frequently involved were the axial skeleton (5/88, 5.7%) or the other long bones (13/88, 14.8%). One tumor was located in the calcaneus. Histological subtype was conventional OS in 79 cases (including 17 chondroblastic, 2 fibroblastic and 6 unusual histological subtypes), telangiectatic OS in 8 cases and small cell OS in 1 case.

There was no significant difference in overall survival (measured as survival since development of metastases to correct for time dependency of the variable) between patients with synchronous and metachronous pulmonary disease (group A vs. B *P*=0.16). When dividing the 62 patients with

Table 3.1 Overview of patients with pulmonary metastases of high grade osteosarcoma under age 40, treated from 1990 to 2008

	Pulmonary metastases		
	Synchronous (group A)	Metachronous (group B)	Total
No metastasectomy for pulmonary metastases *(group 1)*	*1A*, 5 (5.7%)	*1B*, 27 (30.7%)	32 (36.4%)
Metastasectomy for pulmonary metastases *(group 2)*	*2A*, 21 (23.9%)	*2B*, 35 (39.8%)	56 (63.6%)
Total	26 (29.5%)	62 (70.5%)	88

metachronous lung metastases (group B) into three equal groups of 21 patients each based on the duration of the disease-free interval, longer disease-free interval was associated with better survival, with most deaths occurring in patients in the first tertile (metastasis from day 1 to day 310 since diagnosis, Fig. 3.1) (*P*=0.02). Other factors associated with poor overall survival for patients with pulmonary metastases were higher numbers of pulmonary nodules as determined by CT scanning (9.3% increase in hazard for each additional pulmonary nodule, *P*=0.001) and bilateral involvement (*P* logrank=0.008). Patients with bone or other metastases present at the time of diagnosis of the pulmonary metastases had worse overall survival, mainly because in these cases, resection of metastases (pulmonary and others) was often not performed. Histological subtype, histological response to neo-adjuvant chemotherapy in the primary tumor (<10% viable tumor), location of the primary tumor, age at diagnosis, occurrence of local relapse and year of diagnosis did not affect survival in this cohort of patients with pulmonary metastasis.

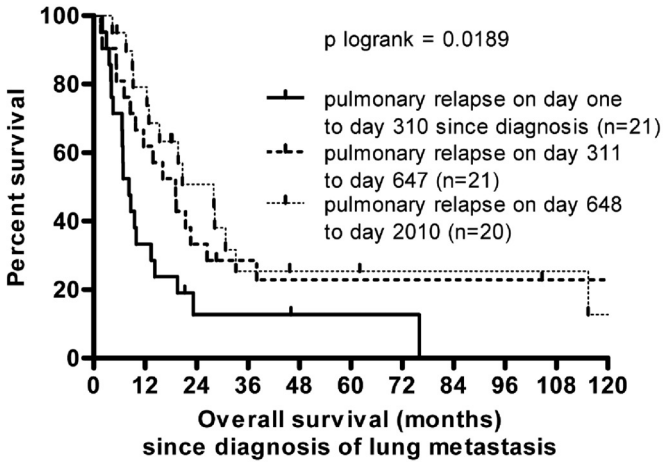


Fig. 3.1 Kaplan–Meier curve of patients with metachronous pulmonary metastases of osteosarcoma (group B), divided into three equal groups based on the duration of the disease-free interval (tertiles), demonstrating worse overall survival for patients with pulmonary relapse from day 1 to day 310 of diagnosis (solid line, *P* logrank=0.02).

Resection of pulmonary metastases

The majority of patients with pulmonary metastases were treated surgically for these metastases at least once (56/88, 63.6%). Overall survival of these patients (group 2) was significantly better than of patients ineligible for metastasectomy (group 1, Table 3.1 and Fig. 3.2, $P<0.0001$). Irresectability of disease as determined in multidisciplinary meetings including radiologists, pathologists, thoracic and orthopedic surgeons and clinical oncologists was the reason not to perform metastasectomy in most cases (81.3%) (Table 3.2). One patient with radiological evidence of lung metastases who did not undergo metastasectomy is a long-term survivor (duration of follow-up 18 years). In this patient, three pulmonary nodules appeared on CT-scanning 1 year after diagnosis of the primary tumor, ranging in size from 0.5 to 2 cm. The largest nodule had a high density, suggesting calcification. In the following year, lesions progressed in both size and number, after which the lesions stabilized without further treatment. All other patients with clinicoradiological evidence for lung metastases who did not undergo metastasectomy died. In these other cases with unresectable disease, no other curative treatments were available; some patients received palliative chemo- or radiotherapy.

Patients not undergoing metastasectomy for pulmonary metastases (group 2) had more nodules (mean of six vs. three, $P=0.002$), more often had bilateral disease (25% vs. 46%, χ^2 $P=0.06$) and more often poor histological response to neo-adjuvant chemotherapy in the primary tumor (78% vs. 64%, χ^2 $P=0.05$) than patients eligible for surgery (group 1). There was no difference in age.

Male sex, higher numbers of pulmonary metastases and viability of resected metastases are independent risk factors after surgical treatment of pulmonary metastases (group 2)

Although patients who were selected for pulmonary metastasectomy (group 2) had improved survival (Fig. 3.2), overall survival was still poor at about 23%. The majority of patients (30/56) underwent thoracotomy just once, but there was no significant survival difference for patients undergoing metastasectomy once or more often ($P=0.29$). This is also reflected in Fig. 3.3, which demonstrates the possibility of achieving complete remission after repeated metastasectomies. Males had surgery for metastases more often than females (70% vs. 48%, χ^2

Table 3.2 Reasons not to undergo surgical removal of pulmonary metastases

Number of patients (%)	Reason not to undergo pulmonary metastasectomy
26/32 (81.3%)	unresectable disease (including metastases to other sites (n=6) and rapidly progressive disease during neo-adjuvant chemotherapy (n=5))
1/32 (3.1%)	died before metastasectomy could be performed
2/32 (6.3%)	physical or mental condition of the patient did not allow it
1/32 (3.1%)	unknown
2/32 (6.3%)	regression of pulmonary metastasis during (neo-)adjuvant chemotherapy (one patient still alive after 18 years follow up, one patient had unresectable relapse in other organs two years later and died of disease)

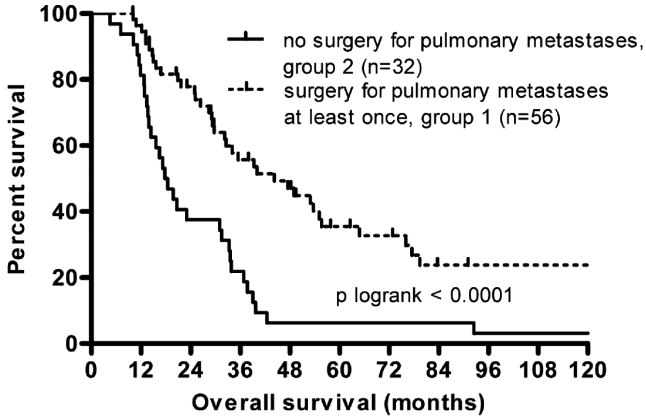


Fig. 3.2 Kaplan–Meier curve comparing overall survival of patients with pulmonary metastases treated surgically (group 1) and nonsurgically (group 2). Patients not treated surgically have a significantly worse overall survival (solid line, *P* logrank<0.0001).

P=0.05), but had worse overall survival (Fig. 3.4, *P*=0.04). Almost all of the patients with pleural disruption evident on histological examination or incomplete resection of the metastases in at least one of the metastasectomies died of disease, but this failed to reach significance (*P*=0.28). Twenty-nine patients (51.8%) had chemotherapeutic treatment before at least one of the metastasectomies. This chemotherapeutic treatment was given to patients either as a part of their primary (neo-) adjuvant treatment or to patients presenting with an initially unresectable pulmonary recurrence during followup. Nine patients had completely necrotic metastases removed at least once (as determined by histological examination) and there was a trend for better survival in these patients (*P*=0.09). Whether or not additional treatment was given before metastasectomy, did not influence survival, but there was an association between premetastasectomy treatment and subsequent removal of necrotic metastases (χ^2 *P*=0.04).

As was the case in the entire cohort of patients with pulmonary metastases, higher numbers of metastases visible on CT scan prior to metastasectomy was associated with worse survival in group 2 patients (who underwent metastasectomy at least once, *P*=0.04). Similarly, there was a trend for patients with bilateral disease to have worse overall survival (*P*=0.07). However, 4 of 15 patients with 5 or more nodules on CT scan did survive after resection of these lesions (follow-up since first metastasis 18–38 months). There was a reasonable correlation between number of metastases as estimated by CT scan and the number of metastases found at metastasectomy (r^2 0.41, slope 0.7, *P*<0.0001), which did not change during the study period. Factors not associated with outcome were histological subtype of the primary tumor, histological response in the primary tumor to neo-adjuvant chemotherapy, occurrence of local relapse, local resection or amputation of the primary tumor and age at diagnosis. In contrast to analysis performed on the entire cohort of 88 patients, disease-free interval was not associated with prognosis in this subset of 56 patients who underwent surgery for pulmonary metastases.We used multivariate

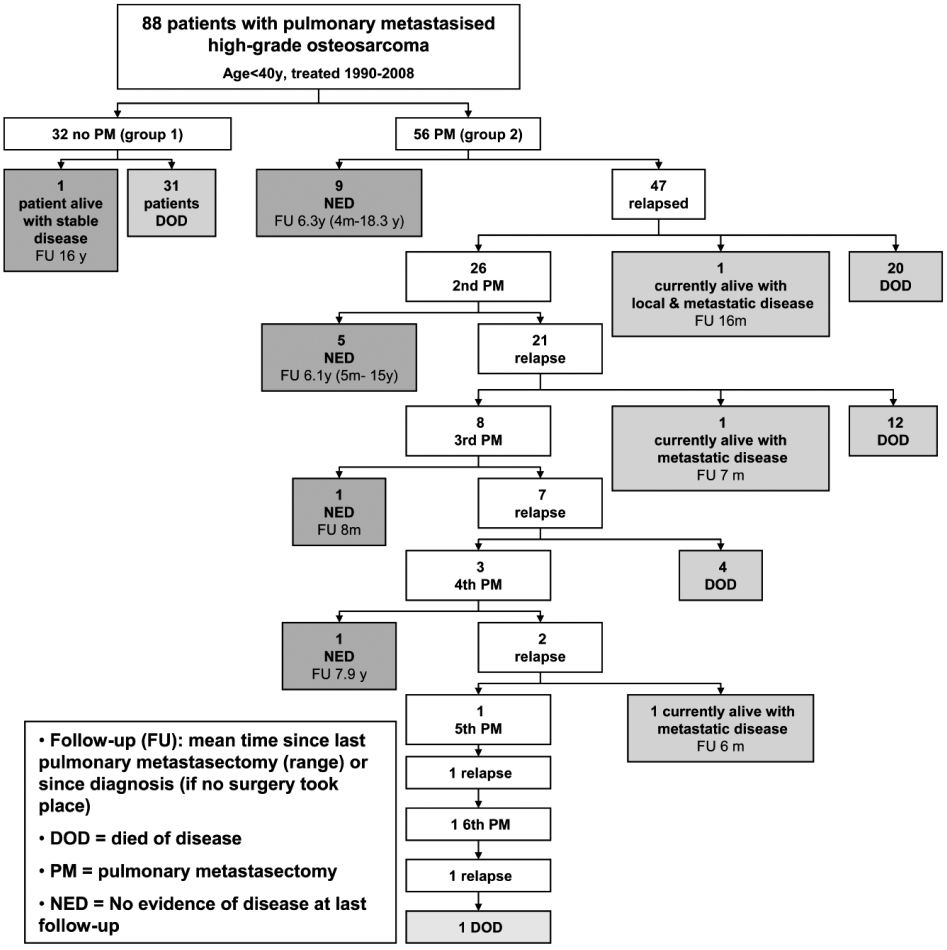


Fig. 3.3 Flow chart of all patients, demonstrating the possibility of achieving complete remission after repeated surgery for pulmonary metastases (including a patient who received surgery for pulmonary metastases four times and is still in complete remission almost 8 years since the last surgery).

Cox regression analysis of risk factors in group 2 patients who underwent metastasectomy at least once, entering only variables with a *P*-value lower than 0.10 as determined by univariate analysis. This revealed male sex of the patient, higher numbers of pulmonary metastases and viability of resected metastases to be independent risk factors for worse outcome in pulmonary metastasized OS patients treated surgically (Table 3.3).

DISCUSSION

In this cohort of patients with pulmonary metastasized high-grade OS, 26/88 patients had clinically detectable pulmonary metastases at diagnosis (group A, Table 3.1) and 62/88 relapsed

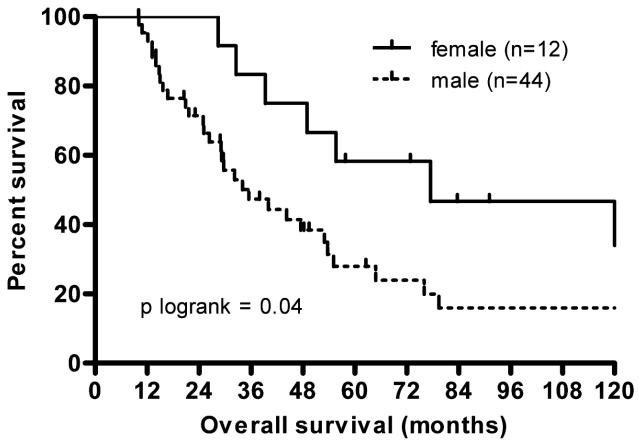


Fig. 3.4 Kaplan–Meier curve of survival after metastasectomy (group 2 patients) for males (dashed line) versus females (solid line). Males had surgery for metastases more often than females (70% vs. 48%, $\chi^2 P$=0.05), but had worse overall survival (*P* logrank=0.04, analysis on subgroup who underwent surgery for pulmonary metastatic disease).

Table 3.3 Multivariate Cox regression analysis to determine independent risk factors for survival in patients surgically treated for pulmonary metastasized high-grade osteosarcoma (group 2 patients)

Risk factor	*P*-value	Hazard ratio
Female sex	0.05	0.409
Completely necrotic metastases	0.03	0.167
Increasing numbers of metastases	0.04	1.293

with pulmonary metastases (group B). Metastasectomy was almost invariably required for cure, as has been previously described by others [12;97;121]. Since pulmonary metastasectomy is a safe and effective treatment, both in pediatric and adult patients, metastasectomy has become standard of care for patients with pulmonary metastasized OS when these metastases are deemed resectable and there are no other contra-indications for surgery (including metastasis to other sites) [261;269]. In our well-defined cohort of patients with detailed and extensive follow-up data we were able to confirm important risk factors determining survival in OS patients with pulmonary metastases, that is, extent of disease and longer disease-free interval. In addition, we have identified the novel independent risk factors male sex and resection of metastases containing viable tumor cells.

The administration of chemotherapeutic agents before metastasectomy is associated with a subsequent higher chance of resecting necrotic metastases, which in turn is associated with better overall survival. This would suggest a potential beneficial role of second-line chemotherapeutic agents in the treatment of metastasized OS, even when these lesions are deemed resectable. However, since we did not find a direct association between chemotherapeutic treatment and overall survival in the group of patients surgically treated

for pulmonary metastases, the addition of chemotherapy to the surgical treatment of patients with pulmonary metastases remains unproven. In previously published studies it has also been difficult to establish what the value of second-line chemotherapeutic treatment is to the surgical management of metastasized OS [23]. This could either mean that the true benefit is very small, or that the treatments employed are too heterogeneous to draw definite conclusions.

As was found in other studies, large metastatic tumor burden (defined as five or more pulmonary nodules or bilateral involvement), has prognostic relevance. It is now well known that spiral CT scanning (1mm slices) is more sensitive than conventional CT, allowing the detection of significantly larger number of nodules and also smaller nodules <5mm in diameter. Even these small pulmonary nodules should be regarded as probable pulmonary metastases when other risk factors for pulmonary nodules, such as smoking history or prior granulomatous disease, are absent. The proportion of patients diagnosed with pulmonary metastases in this study did not change during the study period. Previously, Kayton et al. [120] reported that CT scanning of the chest underestimates the number of metastatic lesions in OS. In our cohort there was a reasonable correlation between number of metastases as predicted by CT scan and number of metastases as determined at resection. However, we feel that the presence of high numbers of pulmonary nodules should not guide decisions regarding resection if the nodules are resectable; a third of patients with high tumor burden, that is, five or more nodules onCT scan prior to surgery, survive after resection of the lesions with a median duration of follow-up of 25 months. Similarly, patients experiencing relapse after first metastasectomy can still benefit from repeated metastasectomies if these lesions are resectable and if there is no risk of respiratory compromise.

The reason for the relationship with gender and outcome in our cohort is unclear. There was a male predominance in our cohort (71.6% males vs. 28.4% females). Males had their metastases significantly more often surgically resected than females. However, men had lower overall survival even after resection of metastases. A recently published review of OS incidence and survival rates from 1973 to 2004 in the United States noted a worse overall survival for males over all age groups, concordant with previous smaller studies [177;205;227]. In another study, a strong correlation between male sex and poor histological response to pre-operative chemotherapy was found, although this did not result in worse overall survival [24]. OS cells express sex steroid receptors and it has been observed that 2-methoxyestradiol (2-ME), a naturally occurring metabolite of 17beta-estradiol (E2), induces cell death in OS cells [57;155]. It remains unclear whether direct effects of sex steroids on neoplastic cells play a role in the observed better outcome for females, or if other mechanisms are underlying this observation.

Patients with OS which has a good histological response of the primary tumor to neo-adjuvant chemotherapy have better overall survival [98]. In our cohort of patients with OS and metastatic disease (group A and group B), there was no association between response to chemotherapy and survival. Similarly, the previously reported association of chondroblastic tumors with good histological response to chemotherapy and better overall survival, was not present in this cohort of patients with OS and lung metastasis. However, when analyzing all patients diagnosed with OS in our center, including those that did not develop pulmonary metastases the association between poor histological response and poor survival was

also present in our cohort. This suggests that poor histological response to chemotherapy probably determines poor survival through risk of developing clinically detectable, pulmonary metastases. However, once these metastases have developed, the histological response in the primary tumor was no longer relevant in our series.

Analysis of patients with resectable non-metastatic OS treated in three consecutive EOI trials demonstrated the association between early recurrence and poor survival [83]. In the current study, this association was also present in the subgroup of patients presenting with metachronous pulmonary metastases, but this association disappeared when patients eligible for surgical treatment of the metastases were selected. This indicates that patients presenting with relapse roughly within the first year after diagnosis have a higher chance of presenting with unresectable pulmonary involvement, but treatment of resectable pulmonary disease results in a similar outcome in this group.

In conclusion, this well-defined cohort of OS patients with pulmonary metastases treated within a single institution allowed us to establish that higher number of pulmonary nodules, resection of vital metastases and male sex were associated with poor overall survival. In the present study we confirm extent of disease, that is, number of pulmonary nodules, to be an important independent risk factor determining survival in OS patients with pulmonary metastases. In addition we demonstrate that female sex and resection of necrotic metastases are associated with better survival after pulmonary metastasectomy. Importantly, we demonstrate that even after repeated metastasectomies, cure can be achieved in a subset of patients.

ACKNOWLEDGMENT

We wish to thank V.L. van Voorst for help in data management. E.P.B. is funded by a grant from The Netherlands Organization for Health Research and Development (ZonMW grant 920-03-399). The Leiden University Medical Center is partner of EuroBoNet, a European Commission granted network of excellence for studying the pathology and genetics of bone tumors. E.P.B. and J.K.A. contributed equally to this work.

4.

Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage-activating agents

Emilie P. Buddingh, Marieke L. Kuijjer, Ronald A.J. Duim, Horst Bürger, Konstantin Agelopoulos, Ola Myklebost, Massimo Serra, Fredrik Mertens, Pancras C.W. Hogendoorn, Arjan C. Lankester, Anne-Marie Cleton-Jansen

Clin Cancer Res. 2011 Apr 15;17(8):2110-9.

ABSTRACT

PURPOSE: High-grade osteosarcoma is a malignant primary bone tumor with a peak incidence in adolescence. Overall survival of patients with resectable metastatic disease is approximately twenty percent. The exact mechanisms of development of metastases in osteosarcoma remain unclear. Most studies focus on tumor cells, but it is increasingly evident that stroma plays an important role in tumorigenesis and metastasis. We investigated the development of metastasis by studying tumor cells and their stromal context. EXPERIMENTAL DESIGN: To identify gene signatures playing a role in metastasis, we performed genome-wide gene expression profiling on prechemotherapy biopsies of patients who did (n=34) and patients who did not (n=19) develop metastases within five years. Immunohistochemistry was performed on pre-treatment biopsies from two additional cohorts (n=63 and n=16), and on corresponding post-chemotherapy resections and metastases. RESULTS: 118/132 differentially expressed genes were upregulated in patients without metastases. Remarkably, almost half of these upregulated genes had immunological functions, particularly related to macrophages. Macrophage associated genes were expressed by infiltrating cells and not by osteosarcoma cells. Tumor-associated macrophages (TAMs) were quantified with immunohistochemistry and were associated with significantly better overall survival in the additional patient cohorts. Osteosarcoma samples contained both M1 (CD14/HLA-DRα positive) and M2 type TAMs (CD14/CD163 positive and association with angiogenesis). CONCLUSION: In contrast to most other tumor types, TAMs are associated with reduced metastasis and improved survival in high-grade osteosarcoma. This study provides a biological rationale for the adjuvant treatment of high-grade osteosarcoma patients with macrophage-activating agents, such as muramyl tripeptide.

INTRODUCTION

High-grade osteosarcoma is a malignant bone tumor characterized by the production of osteoid. The highest incidence is in adolescent patients, with a second peak in patients over 40 years of age [214]. Despite wide-margin surgery and intensification of chemotherapeutic treatment, overall survival rates have reached a plateau at about sixty percent [13;24;141]. Novel treatment modalities are needed, but data on critical biological mechanisms allowing the development of novel therapeutic agents are scarce for this relatively rare tumor. In addition to conventional chemotherapeutic agents, recent trials have explored immunostimulatory strategies. The ongoing EURAMOS-1 trial randomizes for treatment with interferon (IFN)-α in patients with good histological response to neo-adjuvant chemotherapy [160]. A recently published clinical trial has shown improved overall survival for osteosarcoma patients treated with the macrophage activating agent muramyl tripeptide (MTP) added to the standard chemotherapy regimen [170]. However, only limited information on macrophage infiltration and activation in osteosarcoma is available [126].

Tumor-associated macrophages (TAMs) may promote tumorigenesis through immunosuppression, expression of matrix-degrading proteins and support of angiogenesis. In numerous cancer types, high numbers of M2 or 'alternatively activated' TAMs are associated with a worse prognosis [93;112;138;146;266;273]. M2 macrophages have important functions in wound-healing and angiogenesis, express high levels of the immunosuppressive cytokines IL-10 and TGF-β and express scavenger receptors such as CD163 [210;237]. 'Classical activation' of macrophages by interferon-γ or microbial products results in polarization towards M1 type macrophages. M1 macrophages express high levels of pro-inflammatory cytokines such as interleukin (IL)-12 , IL-1, and IL-6 and have potent anti-tumor efficacy, both by reactive oxygen species and cytokine-induced cytotoxicity and by induction of natural killer (NK) and T cell activity [188]. Rarely, high numbers of TAMs are associated with better prognosis [73;122]. In these cases, TAMs are presumably polarized towards an M1 phenotype, although macrophage subtypes were not reported in these two studies. Alternatively, macrophages may directly phagocytose tumor cells, as has been demonstrated in acute myeloid leukemia [109].

To investigate the role of stroma and stroma-tumor interactions important in metastasis of osteosarcoma, we investigated the development of metastasis by studying tumor cells and their stromal context. Using genome-wide expression analysis, we showed that high expression of macrophage-associated genes in pre-treatment biopsies was associated with a lower risk of developing metastases. In addition, we quantified and characterized TAMs in two independent cohorts, including pre-treatment biopsies, post-chemotherapy resections, and metastatic lesions. In contrast to the tumor-supporting role for TAMs in most epithelial tumor types, higher numbers of infiltrating TAMs correlated with better survival in osteosarcoma. Our findings suggest that macrophages have direct or indirect anti-osteosarcoma activity and provide a possible explanation for the beneficial effect of treatment with macrophage activating agents in osteosarcoma.

MATERIALS AND METHODS

Patient cohorts

Genome-wide expression profiling was performed on snap-frozen pre-treatment diagnostic biopsies containing viable tumor material of 53 resectable high-grade osteosarcoma patients from the EuroBoNet consortium (http://www.eurobonet.eu) (cohort 1). For immunohistochemical validation a tissue microarray containing 145 formalin-fixed paraffin-embedded (FFPE) samples of 88 consecutive high-grade osteosarcoma patients with primary resectable disease (cohort 2) and 29 FFPE samples of a cohort of 20 consecutive high-grade osteosarcoma patients with resectable disease were used (cohort 3), including material from pre-treatment biopsies, postchemotherapy resections, and metastatic lesions [183]. Clinicopathological details can be found in Suppl. Table 4.1. All biological material was handled in a coded fashion. Ethical guidelines of the individual European partners were followed and samples and clinical data were stored in the EuroBoNet biobank.

Cell lines

The nineteen osteosarcoma cell lines HAL, HOS, HOS-143b, IOR/MOS,IOR/OS10, IOR/OS14, IOR/OS15, IOR/OS18, IOR/OS9, KPD, MG-63, MHM,MNNG-HOS, OHS, OSA, Saos-2, SARG, U2OS, and ZK-58 were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum and 1% Penicillin/Streptomycin (Invitrogen) as previously described [199].

RNA isolation, cDNA synthesis, cRNA amplification, and Illumina Human-6 v2.0 Expression BeadChip hybridization

Osteosarcoma tissue was snap-frozen in 2-Methylbutane (Sigma-Aldrich, Zwijndrecht, the Netherlands) and stored at -70°C. Using a cryostat, 20 µm sections from each block were cut and stained with hematoxylin and eosin to ensure at least 70% tumor content and viability. RNA was isolated using TRIzol (Invitrogen), followed by RNA clean-up using the QIAGEN Rneasy mini kit with on-column DNAse treatment (Venlo, the Netherlands). RNA quality and concentration were measured using an Agilent 2100 Bioanalyzer (Santa Clara,CA, USA) and Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Synthesis of cDNA, cRNA amplification, and hybridization of cRNA onto the Illumina Human-6 v2.0 Expression BeadChips (San Diego, CA, USA) were performed as per manufacturer's instructions. Reverse transcriptase quantative polymerase chain reaction (RT-qPCR) RT-qPCR analysis of selected target genes was performed as previously described [220]. Each experiment was performed in duplicate using an Automated Liquid-Handling System (Tecan, Genesis RSP 100, Männedorf, Switzerland). Data were normalized using geometric mean expression levels of three reference genes, *i.e. SRPR*, *CAPNS1*, and *TBP* using geNorm (http://medgen.ugent.be/~jvdesomp/genorm/). Primer sequences can be found in Suppl. Table 4.2.

Enzymatic and fluorescent immunostainings

Enzymatic and fluorescent immunostainings were performed on 4 µm sections of FFPE tissue as previously described [183]. Details regarding antibodies and procedures can be found in

Suppl. Table 4.3. In case of double immunohistochemistry (IHC), incubation with anti-CD45 and development with DAB+ (Dako, Glostrup, Denmark) occurred first, followed by a second antigen retrieval before incubation with either anti-CD163 or anti-HLA-DRα and development using the alkaline-phosphatase substrate Vector Blue (Vector Labs, Burlingam, CA, USA). In case of double immunofluorescent (IF) stainings, primary antibodies were co-incubated overnight. As a positive control normal and formic acid decalcified tonsil was used, as a negative control no primary antibody was added. Tissue microarray slides were scanned using the MIRAX SCAN slide scanner and software (Zeiss, Mirax 3D Histech, Hungary). Numbers of positively stained cells and vessels were counted using ImageJ (National Institutes of Health, Bethesda, Maryland, USA) and averaged per 0.6 mm core. IF and double IHC images were acquired using a Leica DM4000B microscope (Wetzlar, Germany) fitted with a CRI Nuance spectral analyzer (Cambridge Research and Instrumentation Inc., Woburn, MA, USA) and analyzed using the supplied co-localization tool to determine percentage of single and double positive pixels per region of interest.

Microarray data analysis

Gene expression data were exported from BeadStudio version 3.1.3.0 (Illumina) in GeneSpring probe profile format and processed and analyzed using the statistical language R [211]. As Illumina identifiers are not stable and consistent between different chip versions, raw oligonucleotide sequences were converted to nuIDs [59]. Data were transformed using the variance stabilizing transformation algorithm to take advantage of the large number of technical replicates available on the Illumina BeadChips [145]. Transformed data were normalized using robust spline normalization, an algorithm combining features of quantile and loess normalization, specifically designed to normalize variance stabilized data. All microarray data processing was carried out using Bioconductor package lumi [60;84]. Quality control was performed using Bioconductor package arrayQualityMetrics [119]. MIAME-compliant data have been deposited in the GEO database (www.ncbi.nlm.nih.gov/geo/, accession number GSE21257).

Statistical analysis

Differential expression between patients who did (n=34) and did not (n=19) develop metastases within five years from diagnosis of the primary tumor was determined using Linear Models for Microarray Data (LIMMA),[240] applying a Benjamini and Hochberg False Discovery Rate adjusted *P*-value cut-off of 0.05. Other univariate statistical analyses were performed using GraphPad Prism software (version 5.01, La Jolla, California, USA). Multivariate survival analyses were carried out according to the Cox proportional hazards model in SPSS (version 16.0.2, Chicago, Illinois, USA). Two-sided *P*-values <0.05 were determined to be significant; *P*-values between 0.05 and 0.15 were defined to be a trend.

RESULTS

High expression of macrophage-associated genes in osteosarcoma biopsies of patients who did not develop metastases within five years from diagnosis (cohort 1)

Comparison of genome-wide gene expression in tumors of patients who did and did not develop metastases within five years resulted in 139 significantly differentially expressed (DE) probes, of which 125 corresponded to 118 upregulated and 14 to downregulated genes in patients who did not develop metastases. A summary of DE genes and detailed descriptions of all probes can be found in Table 4.1 and Suppl. Table 4.4, respectively. Two DE genes were specific for macrophages (*CD14* and *MSR1*) and 30/132 of the DE genes were associated with macrophage functions such as antigen processing and presentation (*e.g. HLA-DRA* and *CD74*) or pattern recognition (*e.g. TLR4* and *NLRP3*). Overall, approximately 20% of the upregulated probes corresponded to genes which were associated with macrophage function and development and an additional 25% of the upregulated probes corresponded to genes with other immunological functions, such as cytokine production and phagocytosis. Four genes were selected for validation of the microarray data using RT-qPCR: *CD14*, *HLA-DRA*, *CLEC5A*, and *FCGR2A*. Expression levels as determined by RTqPCR correlated well with expression levels obtained by microarray analysis (Suppl. Fig. 4.1). Metastases-free survival curves of the same cohort, generated using median expression of the probe of interest as a cut-off determining low and high expression, are shown in Fig. 4.1b and in Suppl. Fig. 4.2. Cox proportional hazards analysis revealed expression of macrophage-associated genes *CD14* and *HLA-DRA* to be independently associated with metastasis free survival (Suppl. Table 4.5).

Macrophage-associated genes are expressed by infiltrating hematopoietic cells and not by tumor cells

The most probable source of expression of the differentially expressed macrophage-associated genes were infiltrating immune cells and not osteosarcoma cells. To confirm this, we performed qRT-PCR of *CD14* and *HLA-DRA* on osteosarcoma cell lines (n=19) and biopsies (n=45, a subset of cohort 1). *CD14* and *HLA-DRA* expression was variable in osteosarcoma biopsies, but almost undetectable in cell lines. This indicates that these macrophage-associated genes were not expressed by tumor cells but by infiltrating cells, since only osteosarcoma biopsies contain macrophage infiltrate, whereas RNA from cell lines is exclusively from tumor cells (Fig. 4.1a, *P*-value Mann-Whitney U test <0.0001). In addition, we performed double IHC for the hematopoietic cell marker CD45, which is not expressed by osteosarcoma tumor cells, and the macrophage marker CD163 or the macrophage-associated protein HLA-DRα (Fig. 4.1c). We chose this approach because no reliable osteosarcoma markers are available (1). Our results confirmed that infiltrating, hematopoietic cells were the source of the macrophage-associated gene expression levels. Together, these data show that osteosarcoma tumor cells do not express macrophage-associated genes, neither *in vitro* nor *in vivo*.

Table 4.1 Differentially expressed genes and probes by category comparing high-grade osteosarcoma patients with and without metastases within five years by genome-wide expression profiling (cohort 1). Twenty percent of differentially expressed probes corresponded to genes which are associated with macrophage functions such as antigen processing and presentation or pattern recognition. Twenty-five percent of the upregulated probes corresponded to genes with other immunological functions, such as cytokine production and phagocytosis.

	Higher expression in patients without metastases			Lower expression in patients without metastases		
Category	**Nr of probes**	**Nr of genes**	**Examples**	**Nr of probes**	**Nr of genes**	**Examples**
Pattern recognition receptor or signaling	18	17	*MSR1, CD14, NLRP3, TLR7, TLR8, TLR4, NAIP, IL1B, PYCARD, NLRC4*	0	0	
Immunological	16	15	*CD86, C1QA, LY9, CD37, LY86*	0	0	
HLA class II	12	12	*HLA-DMB, HLA-DRA, CD74, HLADQA1*	0	0	
Hematopoietic cells	11	10	*HMHA1, MYO1G, LST1*	0	0	
Cytokines and cytokine signaling	7	6	*CXCL16, CSF2RA, IFNGR1, IL10RA*	1	1	*MAP2K7*
Metabolism	9	9	*PFKFB2, SLC2A9, CECR1, ALOX5*	0	0	
Fc receptor	6	4	*FCGR2B, FCGR2A, FGL2, PTPN6*	0	0	
Cytoskeleton	5	5	*HCLS1, WAS, IQGAP2*	1	1	*DNAI2*
(An)ion transporters and channels	4	4	*SLCO2B1, SLC11A1*	1	1	*SLC24A4*
AKT pathway	3	3	*PIK3IP1, PKIB*	0	0	
Endocytosis	3	3	*APPL2, NECAP2*	0	0	
Apoptosis, cell cycle control and proliferation	4	4	*TMBIM4, TNFRSF1B, OGFRL1*	1	1	*BCCIP*
Signaling	4	4	*RGS10, MFNG, FHL2, PILRA*	0	0	
Growth hormone signaling	0	0		1	1	*GHR*
Morphogenesis	0	0		1	1	*HOXC4*
Others	7	6	*CUGBP2, CYP2S1, VAV1, GGN*	2	2	*NSUN5, MRPL4*
Unknown	16	16	*VMO1, MICALCL, MS4A6A*	6	6	*NHN1, BRWD1*
Total	125	118		14	14	

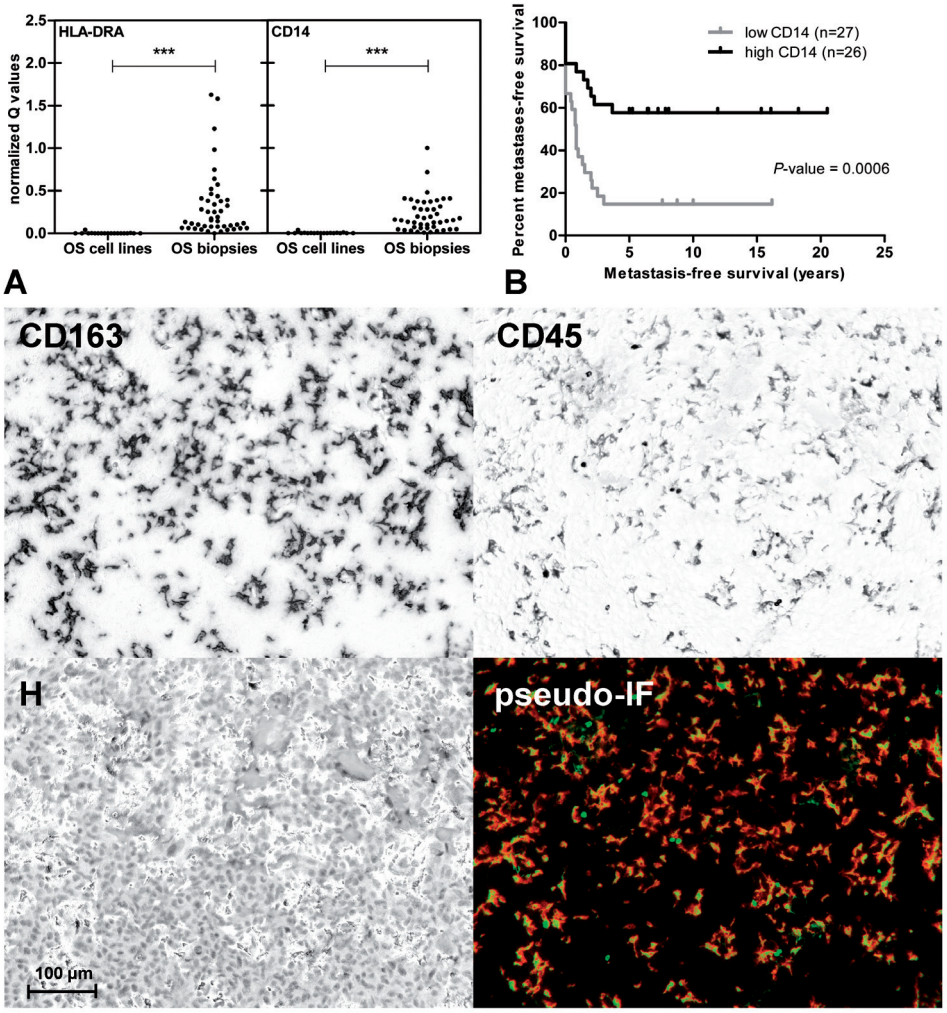


Fig. 4.1 Macrophage-associated genes are not expressed by osteosarcoma tumor cells. **a**, RT-qPCR of osteosarcoma cell lines and biopsies of *CD14* and *HLA-DRA* demonstrating lack of expression by osteosarcoma cells. *P*-value Mann-Whitney U test <0.0001 noted as ***. **b**, high expression of macrophage associated genes was associated with a better metastasis-free survival (cohort 1, Kaplan-Meier curve, *P*-value obtained using logrank test, patients with metastasis at diagnosis have an event at t=0. These patients are included, because patients who develop metastases later on may as well have micrometastases at time of diagnosis. Metastasis-free survival curves for *HLA-DRA*, *CLEC5A*, and *FCGR2A* can be found in Supplemental Figure 2. **c**, double immunohistochemical staining of CD163 with the hematopoietic cell marker CD45 was performed with haematoxylin counterstain (H) and analyzed using spectral imaging microscopy. The pseudo-immunofluorescent image (pseudo-IF) shows CD163 positive cells in red, CD45 positive cells in green and co-localization of both markers in orange. Lack of expression of CD163 and CD45 on surrounding tumor cells (dark blue) and some single positive CD45 cells can be noted.

Macrophage numbers in osteosarcoma biopsies correlate with *CD14* gene expression levels and are positively associated with localized disease and better outcome (cohorts 2 and 3)

To confirm the presence of TAMs in osteosarcoma we stained a tissue microarray containing 145 samples of 88 patients for the macrophage marker CD14 and counted the number of positive cells per tissue microarray core (cohort 2, Fig. 4.2a). CD14 was chosen as opposed to CD68 because the latter marker is not expressed by monocytes and often shows cross-reactivity with mesenchymal tissue (data not shown). Number of CD14 positive cells per tissue microarray core correlated significantly with *CD14* mRNA expression levels (14 samples overlap with gene expression analysis, Spearman correlation coefficient 0.64, *P*-value = 0.01). Similar to the gene expression data, there was a trend for patients with primary localized disease to have higher numbers of macrophages in pre-treatment diagnostic biopsies than patients with metastatic disease at presentation (mean number of macrophages per core 55 *vs*. 27, Mann-Whitney U test *P*-value 0.09). Also, patients with high macrophage counts at diagnosis tended to be less likely to develop metastases within five years (χ^2 *P*-value 0.13). We subdivided this cohort into four quartiles based on numbers of CD14 positive cells in order to determine the group with the best overall survival. No significant differences were found between quartiles 2-4, but patients beloning to this group had better overall survival as compared to patients with low CD14 counts (lowest quartile, or less than 12 CD14 positive cells per tissue array core, Fig. 4.2b, *P*-value log-rank test = 0.02). In another cohort of 16 patients, IF staining of CD14, CD163 and HLA-DRα was performed, again confirming a potential prognostic value of high macrophage numbers (cohort 3, Fig. 4.3, *P*-value log-rank test = 0.01 and Suppl. Fig. 4.3).

Macrophages in osteosarcoma have both M1 and M2 characteristics

To determine the phenotype of macrophages present in osteosarcoma, we performed double IHC with CD14 and either the M1-associated marker HLA-DRα or the M2-associated marker CD163. Not all CD163 and HLA-DR positive infiltrating cells expressed CD14 (Fig. 4.3a and Suppl. Fig. 4.3a). The total number of macrophages as determined by quantifying CD14 positive macrophages was associated with good survival (Fig. 4.3b), but the phenotype of the macrophages (CD14/CD163 double positive versus CD14/HLA-DRα double positive) was not (Suppl. Fig. 4.3b and data not shown). Another M2 characteristic is support of angiogenesis. The number of CD14 positive macrophages correlated with the number of CD31 positive vessels (Fig. 4.2a and Fig. 4.4), but vascularity did not correlate with prognosis (data not shown).

Macrophage numbers in diagnostic biopsies may predict histological response to chemotherapy and macrophage number increases following chemotherapy treatment

There was a trend for high macrophage count (highest three quartiles or >12 CD14-positive cells per tissue array core) in pre-chemotherapy diagnostic biopsies of the primary tumor to predict for good histological response to neoadjuvant chemotherapy (defined as more than 90% non-vital tumor tissue upon final resection), since 46% of patients with high macrophage

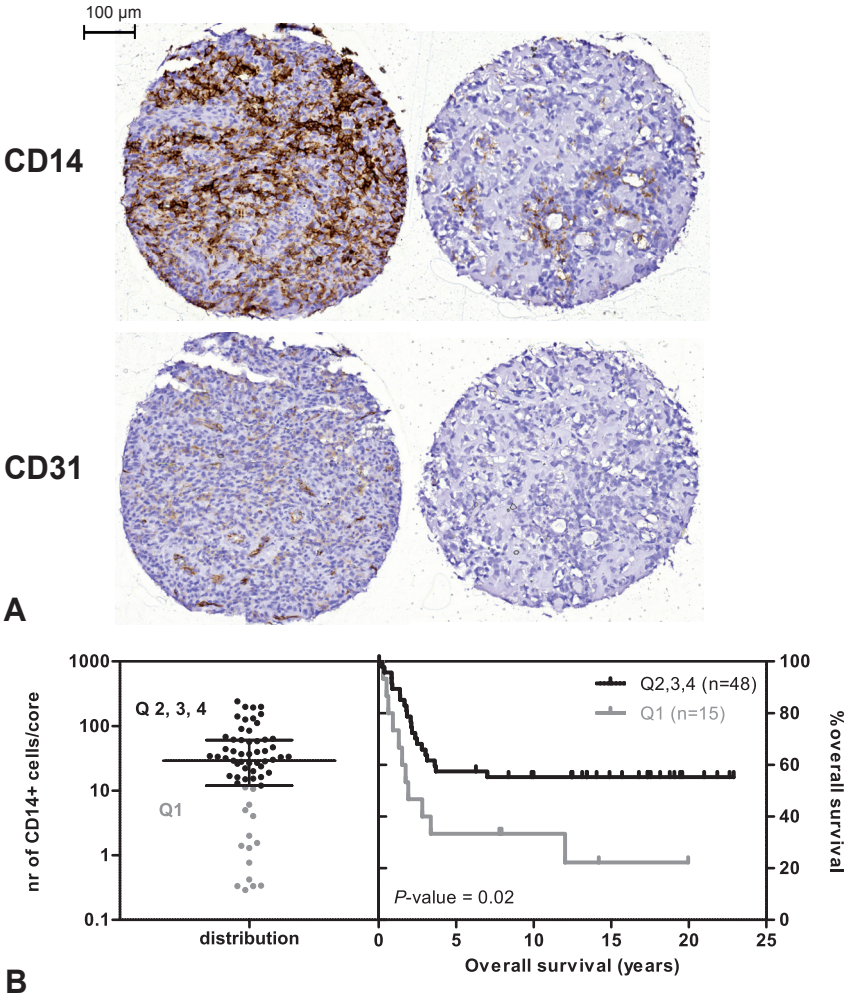


Fig. 4.2 **a**, example of representative stainings of high-grade osteosarcoma with high (left panels) versus low (right panels) levels of macrophage infiltration (CD14 staining) and vascular density (CD31 staining). **b**, high numbers of infiltrating macrophages (left panel, defined as the three upper quartiles, or more than twelve CD14 positive cells per tissue array core) are associated with better overall survival (right panel, *P*-value logrank test = 0.02, cohort 2). Q1, lowest quartile; Q2, 3, 4, three highest quartiles.

numbers and 18% of patients with low macrophage numbers had a good histological response (cohort 2; χ^2 *P*-value 0.09). The prognostic benefit of macrophage counts in osteosarcoma was not independent of histological response using Cox proportional hazard analysis. Macrophage numbers were higher in post-chemotherapy resections of the primary tumor as compared to the pre-chemotherapy biopsies (Suppl. Fig. 4.4). Moreover, gene expression analysis showed upregulation of macrophage-associated probes in post-chemotherapy resections (n=4) as compared with pre-chemotherapy biopsies (n=79, data not shown).

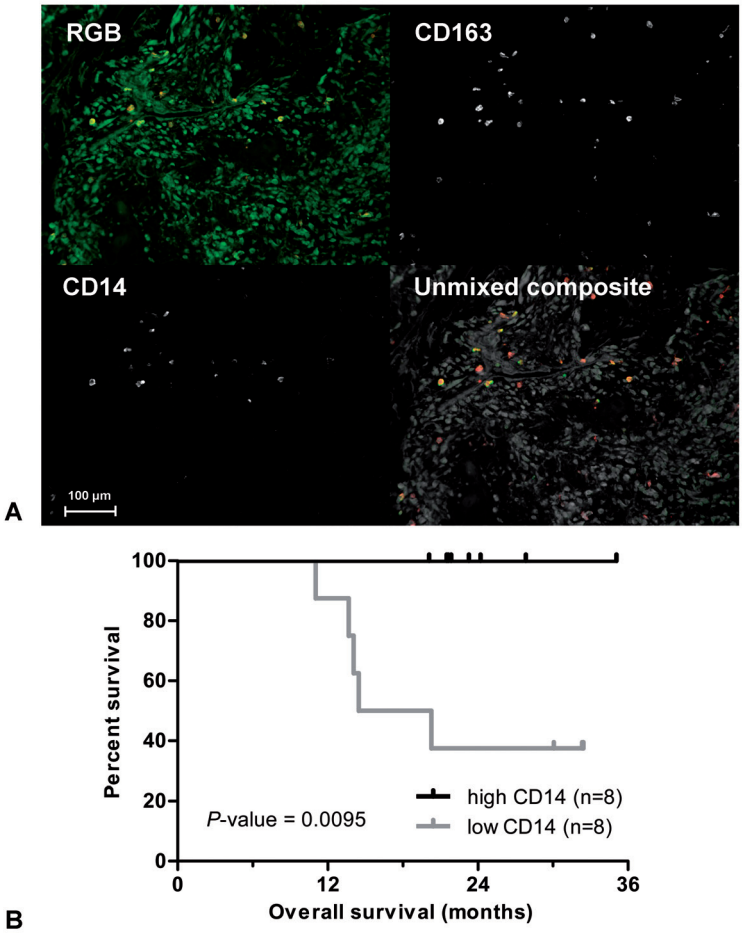


Fig. 4.3 **a**, osteosarcoma samples are infiltrated with CD14 and CD163 single and double positive macrophages. Spectral imaging was used to reduce auto-fluorescence of osteosarcoma cells. In the composite image, CD14 positive cells are represented in green, CD163 positive cells are represented in red, and CD14/CD163 double positive cells are represented in yellow. Background auto-fluorescence of tumor cells is represented in grey. **b**, in an independent cohort of 16 patients (cohort 3), high macrophage infiltration as determined by immunofluorescent CD14 staining was associated with significantly improved overall survival. *P*-values obtained using logrank test, cut-off at the median.

DISCUSSION

Overall survival of high-grade osteosarcoma patients with resectable metastatic disease is poor at about twenty percent [32]. Mechanisms for the development of metastases in osteosarcoma are elusive. To identify genes that play a role in this process, we performed genome-wide expression profiling on pre-chemotherapy biopsies of osteosarcoma patients. We compared patients who developed clinically detectable metastases within five years with patients who did not develop metastases within this time frame (cohort 1). About 20% percent of genes overexpressed in

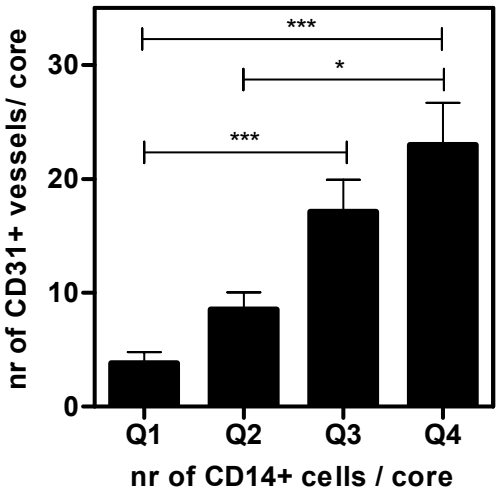


Fig. 4.4 Macrophage infiltration as determined by CD14 positive cell count correlated with vascularity as determined by CD31 positive vessel count. Data of all osteosarcoma samples (pre- and post-treatment primary tumor and metastatic samples, cohort 2) is shown. Q1, lowest quartile; Q2, 3, 4, three highest quartiles. Kruskal-Wallis test $P < 0.0001$, Dunn's post-test P-values <0.05 noted as *, <0.001 noted as ***.

patients without metastases were macrophage-associated, while an additional 25% percent of genes had other immunological functions (for example in phagocytosis, complement activation or cytokine production and response) but could still be attributed to macrophages (Table 4.1 and Suppl. Table 4.4). Thus, in total almost half of the differentially expressed genes belonged to one specific process, *i.e.* macrophage function. Macrophage-associated genes were expressed by infiltrating hematopoietic cells and not by osteosarcoma tumor cells (Fig. 4.1), indicating a possible role for macrophages in preventing metastasis in osteosarcoma. To confirm these findings, we quantified infiltrating macrophages in two additional cohorts (cohort 2 and 3) and found an association with better overall survival in both cohorts.

The anti-metastatic effect of TAMs in osteosarcoma is remarkable, since TAMs support tumor growth in a substantial number of other cancers, which are mostly tumors of epithelial origin. For example, macrophages are associated with the angiogenic switch in breast cancer [144]. We find an association between macrophage infiltration and higher microvessel density, which suggests that the influx of macrophages may support certain aspects of tumor growth in osteosarcoma as well. However, in the case of osteosarcoma, direct or indirect anti-tumor activity of macrophages apparently outweighs their possible tumor-supporting effects. Macrophages can alter their phenotype from M2 to M1 and become the tumor's foe instead of its friend, given the right circumstances [35;92;239]. The TAMs that were identified in this study in osteosarcoma had both M1 and M2 characteristics. The expression of CD163 and the association with angiogenesis are M2 characteristics [144;195]. Some of the differentially expressed genes, such as *MSR1* and *MS4A6A* are specific for M2 macrophages *in vitro* [161]. Others, such as the pro-inflammatory cytokine *IL1B* are more indicative of an M1 phenotype

[188]. How macrophages inhibit osteosarcoma metastasis and if a balance between M1 and M2 type functions is responsible, is unknown.

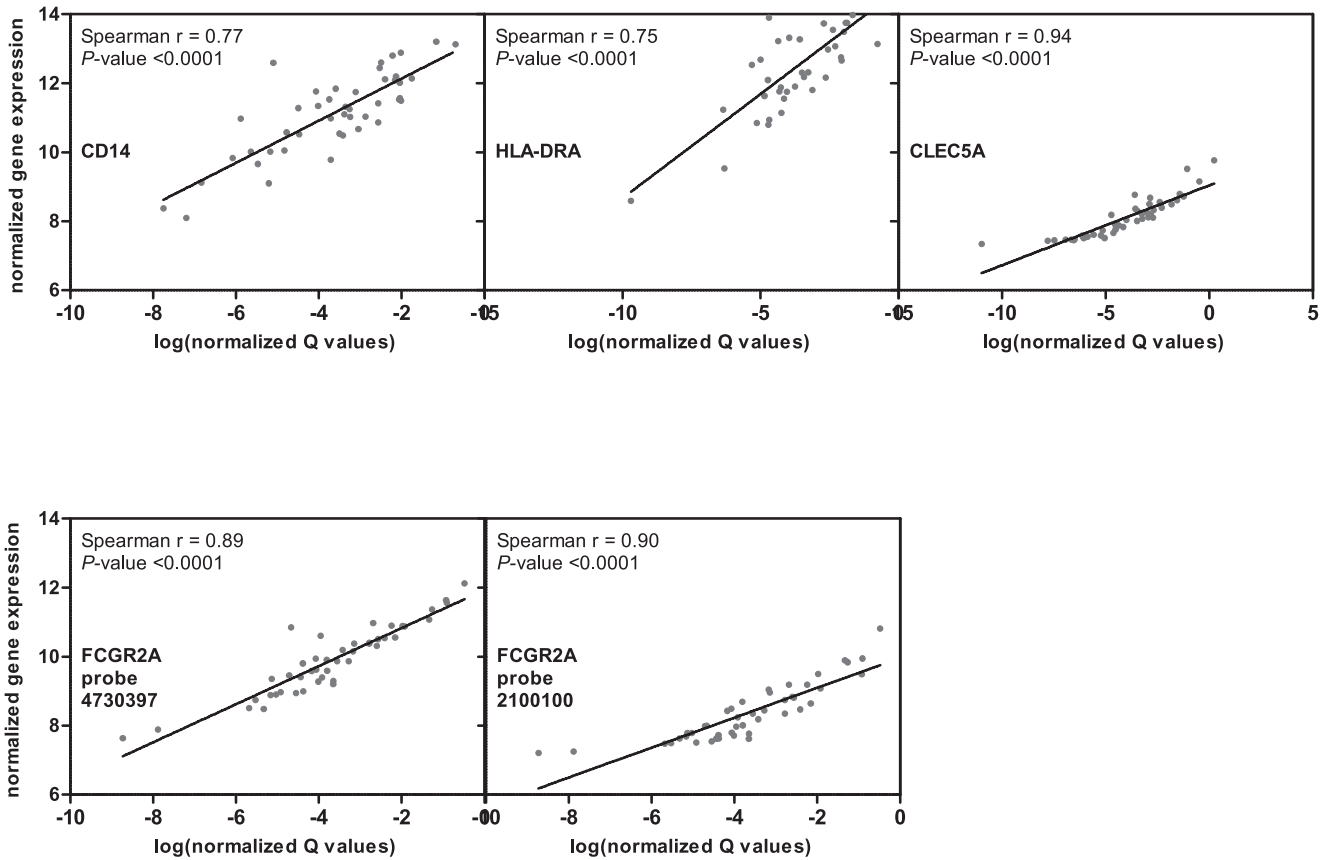
In a multivariate regression model, the survival benefit of high TAM numbers was at least partly dependent on histological response to chemotherapy. Chemotherapy can cause "immunogenic cell death" of cancer cells, resulting in the release of endogenous danger signals [129;288]. The binding of these danger signals to pattern recognition receptors on macrophages can skew polarization of M2 to M1 type TAMs. The interaction between dying tumor cells and resident TAMs may facilitate clearance or inhibit outgrowth of metastatic tumor cells. However, patients with localized disease at diagnosis tended to have a larger macrophage infiltrate than patients with metastatic disease at diagnosis (mean number of macrophages per core 55 *vs*. 27). At this point, patients have not undergone chemotherapeutic treatment yet and an interaction between chemotherapy and macrophages can therefore not be responsible for the anti-metastatic effect of macrophages. Perhaps the anti-metastatic effect of TAMs in these patients is due to the constitutive presence of macrophages with an M1 phenotype. Alternatively, the presence of macrophages might be a reflection of a microenvironment not conducive for metastasis. Although preliminary analysis of a clinical trial investigating the effect of treatment with the macrophage activating agent MTP yielded conflicting results, subsequent analysis revealed that treatment with MTP improved six-year overall survival from 70 to 78% in a cohort of patients with primary localized disease [169;170]. Similar results were obtained in canine osteosarcoma [134]. MTP is a synthetic derivative of muramyl dipeptide (MDP), a common bacterial cell wall component. Muropeptides bind to intracellular pattern recognition receptors of the nucleotide-binding and oligomerization domain (NOD) like (NLR) family, expressed by macrophages [82]. In our study, five genes associated with NLR family signaling and the associated 'inflammasome' were highly expressed in pre-treatment biopsies of patients who do not develop metastases. The differentially expressed genes *NLRP3*, *NAIP*, *NLRC4* and *PYCARD* are components of the inflammasome, *LYZ* is a lysozyme which processes bacterial cell wall peptidoglycan into muramyl dipeptide, a ubiquitous natural analogue of MTP and *IL1B* is the downstream effector cytokine of the inflammasome pathway. Further research is needed to clarify if only patients with high numbers of TAMs benefit from MTP treatment, or if MTP treatment is effective regardless of macrophage number or activation status pre-treatment. Also, it is unknown if treatment with agents promoting macrophage migration or with other macrophage activating agents like toll-like receptor ligands or interferons have a similar beneficial effect on outcome.

Previous genome-wide expression profiling studies in osteosarcoma focused on identifying genes that predict histological response to neo-adjuvant chemotherapy [153;176;194;229]. As a consequence, the importance of macrophages in controlling metastases was not recognized. However, we previously compared gene expression profiles of osteosarcoma biopsies and cultured mesenchymal stem cells and determined which genes are expressed by tumor stroma and not by tumor cells [47]. There is considerable overlap between the stromal genes identified in our previous study and the macrophage-associated genes identified in the present study (including HLA class II genes as the most prevalent differentially expressed group of genes and the macrophage-associated genes *MSR1*, *MS4A6A*, and *FCFGR2A*).

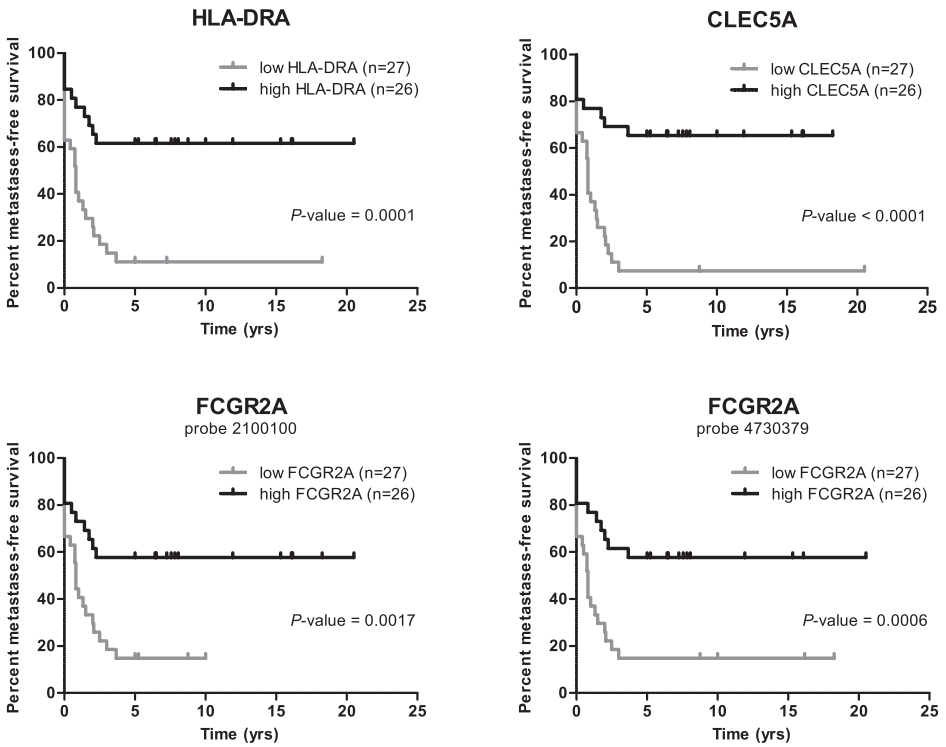
In conclusion, we demonstrated the presence and clinical significance of TAMs in pre-treatment samples of high-grade osteosarcoma. TAMs in osteosarcoma are a heterogeneous cell population with both M1 anti-tumor and M2 pro-tumor characteristics. Although the exact mechanism by which macrophages exert their anti-metastatic functions is still unknown, this study provides an important biological rationale for the treatment of osteosarcoma patients with macrophage activating agents.

ACKNOWLEDGMENT

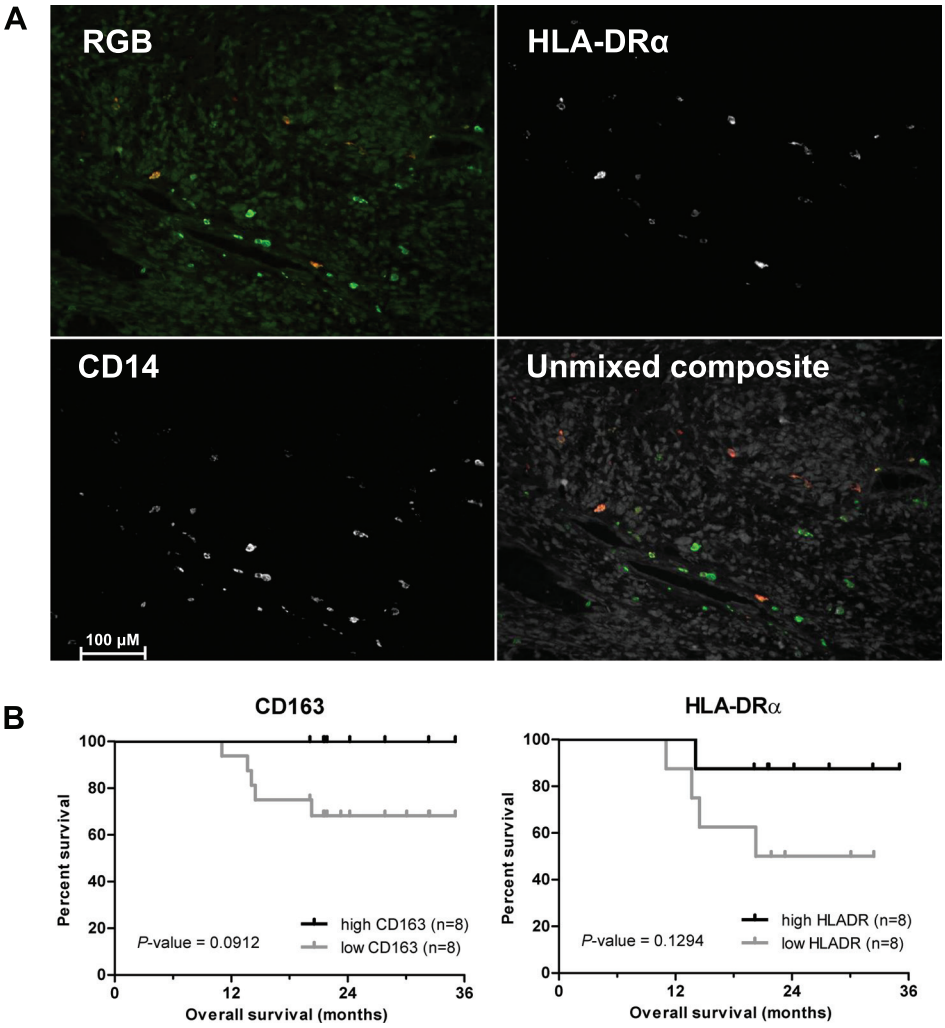
The authors wish to thank Alex Mohseny for culturing the OS cell lines and isolating RNA, Esther Hauben for histological review of all OS specimens used for genome-wide gene expression profiling, Stefan Bielack and Matthias Kevirc for collecting material and clinical data of the samples provided by the University of Münster, Germany, Inge Briaire-Bruijn for technical assistance and Jan Oosting and Eberhard Korsching for discussion on biostatistics and microarray data analysis. This work was supported by EuroBoNet, a European Commission granted Network of Excellence for studying the pathology and genetics of bone tumors (grant number LSHC-CT-2006-018814), by the Netherlands Organization for Health Research and Development (ZonMw, grant number 92003-399 to E.P.B.), and by the Dutch Cancer Society (KWF, grant number 2008-4060 to M.L.K.). E.P.B. & M.L.K. and A.C.L. & A.M.C.J. contributed equally to this study.



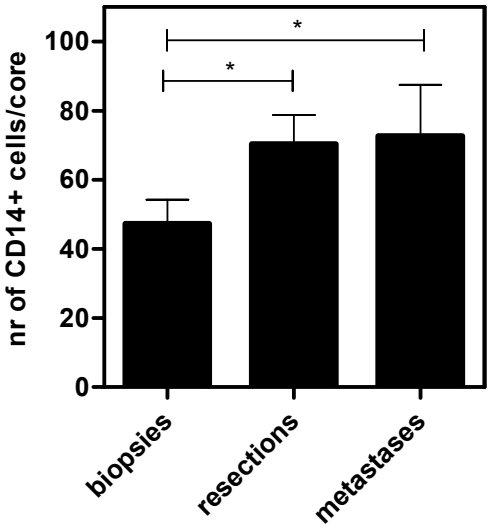
Suppl. Fig. 4.1 Gene expression levels of *CD14*, *HLA-DRA*, *CLEC5A*, and *FCGR2A* as obtained using genome-wide expression profiling correlated well with gene expression levels as determined using RT-qPCR.



Suppl. Fig. 4.2 High levels of *HLA-DRA*, *CLEC5A* and *FCGR2A* as determined using genome-wide expression profiling was associated with significantly improved metastases free survival. *P*-values obtained using logrank method, cut-off at the median (cohort 1).



Suppl. Fig. 4.3 **a**, osteosarcoma samples are infiltrated with CD14 and HLA-DRα single and double positive macrophages. Spectral imaging was used to reduce auto-fluorescence of osteosarcoma cells. In the composite image, CD14 positive cells are represented in green, HLA-DRα positive cells are represented in red, and CD14/HLA-DRα double positive cells are represented in yellow. Background auto-fluorescence of tumor cells is represented in grey. **b**, in 16 patients treated according to the EURAMOS-1 protocol (cohort 3), there was a trend for high macrophage infiltration as determined by immunofluorescent HLA-DRα and CD163 to be associated with improved overall survival. *P*-values obtained using logrank test, cut-off at the median.



Suppl. Fig. 4.4 In post-chemotherapy samples, macrophage numbers increased (post-chemotherapy resections of the primary tumor *vs.* pre-treatment diagnostic biopsies, cohort 2). Kruskal-Wallis test *P*-value = 0.0094, Dunn's post-test *P*-value <0.05 noted as *.

Suppl. Table 4.1 Clinicopathological data of osteosarcoma samples and patient characteristics. Treatment regimens containing high-dose methotrexate (M), doxorubicin (A), cisplatin (P) and/or ifosfamide (I) were used.

Patient characteristics	Cohort 1	Cohort 2	Cohort 3
Samples used for:	Microarray analysis	Tissue microarray: CD14 and CD31 staining	Immunofluorescent staining of CD14, CD163, HLA-DRα
Total nr of samples	53 (of 53 patients)	145 (of 88 patients)	29 (of 20 patients)
pre-treatment biopsies of primary tumor	53 (of 53 patients)	73 (of 73 patients)	16 (of 16 patients)
post-chemotherapy resections of primary tumor	0	45 (of 45 patients)	13 (of 13 patients)
metastatic lesions	0	24 (of 15 patients)	0
Year of diagnosis	1986-2006	1984-2003	2007-2008
Institution			
LUMC, Netherlands	27	all	all
IOR, Italy	7		
LOH, Sweden	2		
Radiumhospitalet, Norway	1		
WWUM, Germany	16		
Treatment regimens	MAPI, MAP, PIA, AP	AP, PIA	MAP
Location of primary tumor	n (%)	n (%)	n (%)
Femur	27 (50.1)	45 (51.1)	18(62.1)
Tibia/fibula	17 (32.1)	31 (35.2)	6 (20.7)
Humerus	8 (15.1)	10 (11.4)	5 (17.2)
Axial skeleton	0 (0)	1 (1.1)	0 (0)
Hand	0 (0)	1 (1.1)	0 (0)
Unknown/ other	1 (1.9)	0 (0)	1 (3.4)
Histological subtype			
Conventional osteosarcoma			
- osteoblastic	32 (60.4)	59 (67.0)	19 (65.5)
- chondroblastic	6 (11.3)	9 (10.2)	3 (10.3)
- fibroblastic	5 (9.4)	3 (3.4)	1 (3.4)
- unusual	7 (13.2)	8 (9.1)	2 (6.9)
Telangiectatic	3 (5.7)	6 (6.8)	4 (13.8)
High grade surface osteosarcoma	0 (0)	1 (1.1)	0 (0)
Small cell osteosarcoma	0 (0)	2 (2.3)	0 (0)
Histological response to pre-operative chemotherapy in the primary tumor			
Unknown	6 (11.3)	12 (13.6)	1 (3.4)
Poor response	29 (54.7)	48 (54.5)	15 (51.7)
Good response	18 (34.0)	28 (31.8)	13 (44.8)
Sex			
Male	33 (62.3)	47 (53.4)	14 (48.3)

Suppl. Table 4.2 RT-qPCR primer sequences. These sequences have been submitted to the Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/). All PCR products were validated by sequencing.

Gene symbol	Product size (bp)	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
CD14	198	GCCGCTGTGTAGGAAAGAAG	AGGTTCGGAGAAGTTGCAGA
CLEC5A	128	GGCGTTGGATCAACAACTCT	GATCCTGCGGTAGCTGATGT
FCGR2A	80	TATGTCCCAGAAACCTGTGG	GGGAGCAGCTTGACTGTCTG
HLA-DRA	141	TGTAAGGCACATGGAGGTGA	ATAGGGCTGGAAAATGCTGA

Suppl. Table 4.3 Antibodies and conditions used for immunohistochemistry and immunofluorescence.

Antibody	Clone/ catalogue nr	Company	Methods
CD31	Ab-1, clone JC/70A	Neomarkers Fremont, CA, USA	Antigen retrieval (AR): 1 mM EDTA solution (pH 8.0). Secondary antibody (2nd ab): Envision Horse Radish Peroxidase (HRP) anti-mouse (Dako), chromogen DAB+ (Dako, K3468)
CD14	Ab-2, clone 7	Neomarkers	AR: 10mM Tris/ 1 mM EDTA (pH 9.0). 2nd ab: Envision anti-mouse HRP (Dako) followed by DAB+; Alexa Fluor-488 goat anti-mouse IgG2a (Invitrogen)
CD163	NCL-CD163	Novocastra, Newcastle Upon Tyne, England	AR: 10mM Tris/ 1 mM EDTA (pH 9.0). 2nd ab: Envision anti-mouse HRP (Dako) followed by DAB+; Goat-anti-mouse alkaline phosphatase (AP) (Dako, nr) followed by Vector Blue (Vector Labs); Alexa Fluor-647 goat anti-mouse IgG1 (Invitrogen)
HLA-DRα	TAL.1B5	Dako	AR: 10mM Tris/ 1 mM EDTA (pH 9.0).2nd ab: Envision anti-mouse HRP (Dako) followed by DAB+; Goat-anti-mouse (AP) (Dako) followed by Vector Blue; Alexa Fluor-647 goat anti-mouse IgG1 (Invitrogen).

Suppl. Table 4.4 Differentially expressed genes and detailed descriptions including references. Probes with a positive log fold change (logFC) are higher in patients without metastases as compared to patients with metastases within five years. Adjusted *P*-value (adj*P*val) <0.05 determined to be significant.

logFC	adjPVal	Symbol	Description	Keywords	References
0.81	0.0035	*FCGR2B*	IgG FcR with a tyrosine-based inhibitory motif expressed on B lymphocytes, monocytes, neutrophils and myeloid dendritic cells.	Fc receptor, B lymphocytes, monocytes, neutrophils, myeloid dendritic cells	[250]
0.89	0.0038	*CLEC5A*	C-type lectin which binds Dengue virus. CLEC5A is highly expressed in murine neutrophils and macrophages. CLEC5a has a role in osteoclastogenesis.	C-type lectin, pattern recognition receptor, macrophages, neutrophils, osteoclasts, dengue virus	[9;39;106]
1.51	0.0083	*ALOX5AP*	This gene encodes a protein which, with 5-lipoxygenase, is required for leukotriene synthesis. Leukotrienes are arachidonic acid metabolites which have been implicated in various types of inflammatory responses and mediate production of endogenous PPAR-γ-ligands.	Arachidonic acid lipoxygenase, leukotrienes, PPAR- γ ligand production	[refseq] [267]
0.17	0.0110	*RNASE3*	Eosinophil cationic protein expressed mainly by eosinophils with antiviral, antibacterial and cytotoxic properties. Also produced by monocytes.	Eosinophils, cytotoxicity, monocytes	[36;271]
0.59	0.0171	*MSR1*	This gene encodes the class A macrophage scavenger receptors, which include three different types generated by alternative splicing. These receptors are macrophage-specific phagocytic pattern recognition receptors.	Scavenger receptor, pattern recognition, macrophages, phagocytosis	[refseq] [28]
1.12	0.0171	*FCGR2A*	IgG Fc receptor involved in phagocytosis by macrophages and neutrophils	Fc receptor, macrophages, neutrophils, phagocytosis	[refseq]
1.09	0.0173	*SPINT2*	Serine peptidase inhibitor, Kunitz type, 2. The protein inhibits HGF activator which prevents the formation of active hepatocyte growth factor. This gene is a putative tumor suppressor, and mutations in this gene result in congenital sodium diarrhea.	Tumor suppressor, hepatocyte growth factor inhibition	[refseq]
1.22	0.0173	*FGL2*	Fibrinogen-like 2 has immunosuppressive properties and binds FCGR2B and FCGR3	Immunosuppression, Fc receptor signaling	[147]
0.33	0.0173	*OGFRL1*	Opioid growth factor receptor-like 1 is related to OGF, which is a negative regulator of cell proliferation and tissue organization in a variety of processes.	Cell proliferation	[refseq]
0.63	0.0173	*CSF2RA*	The protein encoded by this gene is the alpha subunit of the heterodimeric receptor for colony stimulating factor 2, a cytokine which controls the production, differentiation, and function of granulocytes and macrophages.	Cytokine receptor, granulocytes, macrophages	[refseq]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
0.79	0.0173	*CXCL16*	C-X-C motif ligand 16. Expression of CXCL16 by renal and colon cancer correlates with good prognosis. Associated with atherosclerosis. Chemo-attractant of T cells and macrophages.	Chemokine, macrophages, cancer, T cell	[91;101;162]
1.09	0.0173	*HLA-DOA*	Part of HLA class II alpa chain paralogues found in lysosomes in B cells. Regulates HLA-DM-mediated peptide loading on MHC class II molecules	HLA class II, B cells	[refseq]
1.23	0.0173	*HLA-DMB*	Part of HLA class II beta chain paralogues located in intracellular vesicles. DM plays a central role in the peptide loading of MHC class II molecules by helping to release the CLIP (class II-associated invariant chain peptide) molecule from the peptide binding site. Class II molecules are expressed in antigen presenting cells (APC: B lymphocytes, dendritic cells, macrophages).	HLA class II, B cells, dendritic cells, macrophages	[refseq]
1.14	0.0173	*CD86*	This protein is expressed by antigen-presenting cells (APC: B lymphocytes, dendritic cells, macrophages)., and it is the ligand for two proteins at the cell surface of T cells, CD28 antigen and cytotoxic T-lymphocyte-associated protein 4. Binding of this protein with CD28 antigen is a costimulatory signal for activation of the T-cell. Binding of this protein with cytotoxic T-lymphocyte-associated protein 4 negatively regulates T-cell activation and diminishes the immune response.	B cells, dendritic cells, macrophages, co-stimulation of T cells	[refseq]
0.73	0.0173	*VMO1*	Vitelline membrane outer layer 1 homolog	Unknown	
0.67	0.0173		AGENCOURT_10520654 NIH_MGC_128 Homo sapiens cDNA clone IMAGE:6702588 5, mRNA sequence	Unknown	
1.32	0.0173	*HLA-DMA*	Part of HLA class II beta chain paralogues located in intracellular vesicles. DM plays a central role in the peptide loading of MHC class II molecules by helping to release the CLIP (class II-associated invariant chain peptide) molecule from the peptide binding site. Class II molecules are expressed in antigen presenting cells (APC: B lymphocytes, dendritic cells, macrophages).	HLA class II, B cells, dendritic cells, macrophages	[refseq]
0.11	0.0173		UI-H-EZ1-bbe-e-05-0-UI.s1 NCI_CGAP_Ch2 Homo sapiens cDNA clone UI-H-EZ1-bbe-e-05-0-UI 3, mRNA sequence	Unknown	
0.64	0.0182	*NLRP3*	NLRP3 is a NOD-like receptor, and is a member of the NALP3 inflammasome complex. The inflammasome is a multiprotein complexe that can activates caspase-1 and ultimately lead to the processing and secretion of interleukin (IL)-1beta, IL-18 and IL-33. NLRP3 interacts with the apoptosis-associated speck-like protein PYCARD/ASC, which contains a caspase recruitment domain.	NOD-like receptor, inflammasome, monocyte, macrophage	[74;82]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
0.63	0.0182	*MSR1*	This gene encodes the class A macrophage scavenger receptors, which include three different types generated by alternative splicing. These receptors are macrophage-specific phagocytic pattern recognition receptors.	Scavenger receptor, pattern recognition, macrophages, phagocytosis	[refseq] [28]
0.61	0.0186	*PEA15*	PEA15 is a death effector domain (DED)-containing protein predominantly expressed in the central nervous system, particularly in astrocytes. Ubiquitously expressed, implicated in a number of fundamental cellular functions, including apoptosis, proliferation, and glucose metabolism. PED/PEA-15 is an endogenous substrate for protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CAM kinase II), and Akt.	Apoptosis, proliferation, glucose metabolism, PKC, CAM kinase II, AKT.	[refeq] [68]
0.77	0.0187	*PKIB*	The protein encoded by this gene is a member of the cAMP-dependent protein kinase inhibitor family. PKIB and PKA-C kinase can have critical functions of aggressive phenotype of prostate cancer cells through Akt phosphorylation.	PKC, AKT, kinase inhibitor	[refseq] [45]
0.4	0.0187	*SYTL3*	Synaptotagmin-like 3, function unknown	Unknown	[refseq]
0.44	0.0187	*CUGBP2*	CUG triplet repeat, RNA binding protein 2, members of this protein family regulate pre-mRNA alternative splicing and may also be involved in mRNA editing, and translation.	RNA binding protein, alternative splicing	[refseq]
0.69	0.0215	*CSF2RA*	The protein encoded by this gene is the alpha subunit of the heterodimeric receptor for colony stimulating factor 2, a cytokine which controls the production, differentiation, and function of granulocytes and macrophages.	Cytokine receptor, granulocytes, macrophages	[refseq]
1.84	0.0215	*LYZ*	Lysozyme cleaves bacterial cell wall peptidoglycan, resulting in components that can bind the NOD receptor.	Bacterial wall peptidoglycan, NOD	[refseq], [82]
1.1	0.0215	*FPRL2*	Formyl peptide receptors sense formyl peptides in bacteria and mitochondria. These receptors are expressed on phagocytic cells.	Formyl peptide receptor, pattern recognition receptor, phagocytosis	[171]
0.83	0.0217	*FCGR2B*	IgG FcR with a tyrosine-based inhibitory motif expressed on B lymphocytes, monocytes, neutrophils and myeloid dendritic cells.	Fc receptor, B lymphocytes, monocytes, neutrophils, myeloid dendritic cells	[250]
0.69	0.0246	*PIK3IP1*	phosphoinositide-3-kinase interacting protein 1 is a negative regulator of PI3K and suppresses the development of hepatocellular carcinoma	PI3K, AKT, tumor suppressor	[99]
1.49	0.0246	*HLA-DRA*	HLA-DRA is one of the HLA class II alpha chain paralogues. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells (APC: B lymphocytes, dendritic cells, macrophages).	HLA class II, B cells, dendritic cells, macrophages	[refseq]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
0.85	0.0276	*HLA-DPB1*	HLA-DPB1 belongs to the HLA class II beta chain paralogues. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells (APC: B lymphocytes, dendritic cells, macrophages).	HLA class II, B cells, dendritic cells, macrophages	[refseq]
0.32	0.0276	*KCNK13*	This gene encodes one of the members of the superfamily of potassium channel proteins containing two pore-forming domains. The product of this gene is an open channel that can be stimulated by arachidonic acid.	Potassium channel, arachinodic acid	[refseq]
0.47	0.0276	*LRRC25*	Leucine rich repeat containing 25, high expressed in plasmacytoid dendritic cells and granulocytes.	Dendritic cells, granulocytes	[216]
1.49	0.0276	*CD74*	Major histocompatibility complex, class II invariant chain. May also interact with CXCR4 to bind macrophage migration inhibitory factor.	HLA class II, B cell, dendritic cells, macrophages, macrophage migration, CXCR4	[184;232]
0.71	0.0276	*CTSS*	Cathepsin S is a lysosomal cysteine proteinase that may participate in the degradation of antigenic proteins to peptides for presentation on MHC class II molecules.	Cathepsin, HLA class II, B cells, dendritic cells, macrophages	[refseq]
0.49	0.0276	*PFKFB2*	The protein encoded by this gene is involved in both the synthesis and degradation of fructose-2,6-bisphosphate, a regulatory molecule that controls glycolysis in eukaryotes.	Glycolysis, glucose metabolism	[refseq]
1.47	0.0276	*HLA-DPA1*	HLA-DPA1 is one of the HLA class II alpha chain paralogues. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells (APC: B lymphocytes, dendritic cells, macrophages).	HLA class II, B cells, dendritic cells, macrophages	[refseq]
0.88	0.0276	*CD37*	Member of the transmembrane 4 superfamily. The proteins mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility. Expressed by immune cells, including macrophages and osteoclasts.	Tetraspanin, B cells, macrophages, osteoclasts.	[refseq], [11]
0.55	0.0276	*PILRA*	Control of cell signaling via SHP-1 is thought to occur through a balance between PILRalpha-mediated inhibition and PILRbeta-mediated activation.	Cell signalling, SHP-1	[refseq]
0.81	0.0276	*TLR7*	TLR7 recognizes single stranded RNA. TLR7 is expressed in a variety of different cell types of the immune system such as DCs, B cells, monocytes, NK cells and T cells.	Toll-like receptor, pattern recognition receptor, dendritic cells, B cells, monocytes, NK cells, T cells, ssRNA	[16]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
1.15	0.0276	*FCGR2A*	IgG Fc receptor involved in phagocytosis by macrophages and neutrophils	Fc receptor, macrophages, neutrophils, phagocytosis	[refseq]
0.68	0.0276	*TLR6*	This receptor functionally interacts with toll-like receptor 2 to mediate cellular response to bacterial lipoproteins.	Toll-like receptor, pattern recognition receptor, bacterial lipoproteins	[refseq]
0.55	0.0276	*TM6SF1*	Transmembrane 6 superfamily member 1, expressed in hematopoietic cells and testis, function unknown.	Hematopoietic cells	[37]
0.56	0.0276	*CLEC12A*	The C-type lectin encoded by this gene is a negative regulator of granulocyte and monocyte function.	C-type lectin, pattern recognition receptor, bacteria, monocytes, granulocytes	[refseq]
0.36	0.0276	*LY9*	LY9 belongs to the SLAM family of immunomodulatory receptors and interacts with the adaptor molecule SAP. SLAM family of receptors and SAP family of adaptors play critical roles in lymphocyte development, differentiation, and acquisition of effector functions.	SLAM, SAP, lymphocyte development, immune cell effector function	[refseq], [152]
0.36	0.0276	*SLC2A9*	This gene encodes a member of the SLC2A facilitative glucose transporter family. Members of this family play a significant role in maintaining glucose homeostasis. The encoded protein may play a role in the development and survival of chondrocytes in cartilage matrices.	Glucose transport, chondrocyte development	[refseq]
1.22	0.0276	*AIF1*	This gene is induced by cytokines and interferon. Expressed by activated macrophages and regulates endothelial cell activation, signal transduction, and vasculogenesis.	Cytokines, interferon, endothelial cells, macrophages	[refseq], [257;258]
0.79	0.0277	*TNFRSF1B*	The protein encoded by this gene is a member of the TNF-receptor superfamily. This protein and TNF-receptor 1 form a heterocomplex that mediates the recruitment of two anti-apoptotic proteins, c-IAP1 and c-IAP2, which possess E3 ubiquitin ligase activity. The function of IAPs in TNF-receptor signalling is unknown, however, c-IAP1 is thought to potentiate TNF-induced apoptosis by the ubiquitination and degradation of TNF-receptor-associated factor 2, which mediates anti-apoptotic signals.	TNF-receptor superfamily, apoptosis.	[refseq]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
0.59	0.0280	*LITAF*	Lipopolysaccharide is a potent stimulator of monocytes and macrophages, causing secretion of tumor necrosis factor-alpha (TNF-alpha) and other inflammatory mediators. This gene encodes lipopolysaccharide-induced TNF-alpha factor, which is a DNA-binding protein and can mediate the TNF-alpha expression by direct binding to the promoter region of the TNF-alpha gene. The transcription of this gene is induced by tumor suppresor p53 and has been implicated in the p53-induced apoptotic pathway. Mutations in this gene cause Charcot-Marie-Tooth disease type 1C (CMT1C) and may be involved in the carcinogenesis of extramammary Paget's disease (EMPD).	Pattern recognition signalling, monocytes, macrophages, TNF-alpha, p53-induced apoptotic pathway	[refseq]
0.82	0.0289	*ARHGAP30*	Rho GTPase, They play a critical role in muscle differentiation. The protein encoded by this gene binds GTP and is a member of the small GTPase superfamily. It is involved in endosome dynamics and reorganization of the actin cytoskeleton, and it may coordinate membrane transport with the function of the cytoskeleton.	Rho GTPase, cytoskeleton.	[refseq]
1.6	0.0303	*HLA-DRB4*	HLA-DRB4 belongs to the HLA class II beta chain paralogues. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells (APC: B lymphocytes, dendritic cells, macrophages).	HLA class II, B cells, dendritic cells, macrophages	[refseq]
1	0.0322	*IRF8*	The IRF family proteins bind to the IFN-stimulated response element (ISRE) and regulate expression of genes stimulated by type I IFNs, namely IFN-alpha and IFN-beta. Expression of IRF8 is inducible by IFN-gamma. Its target genes are IL-12 and IL-18.	Interferon	[refseq]
0.17	0.0322	*GPX1*	This gene encodes a member of the glutathione peroxidase family. Glutathione peroxidase functions in the detoxification of hydrogen peroxide, and is one of the most important antioxidant enzymes in humans.	Gluthation peroxidase, antioxidant	[refseq]
0.46	0.0322	*HMHA1*	Rho-like GTPase-activating protein, minor histocompatibility antigen restricted to hematopoietic cells.	Minor histocompatibility antigen, hematopoietic cells, Rho-like GTPase-activating protein	[244]
0.1	0.0332	*OR4K15*	Olfactory receptors interact with odorant molecules in the nose, to initiate a neuronal response that triggers the perception of a smell. The olfactory receptor proteins are members of a large family of G-protein-coupled receptors (GPCR) arising from single coding-exon genes.	G-protein-coupled receptors, olfactory receptor	[refseq]
0.62	0.0332	*RGS10*	Regulator of G protein signaling (RGS) family members are regulatory molecules that act as GTPase activating proteins (GAPs) for G alpha subunits of heterotrimeric G proteins. RGS10A is a key component in the RANKL-evoked signaling pathway for osteoclast differentiation.	G-protein signalling, GTPase activating proteins, osteoclast differentiation	[refseq], [283]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
0.38	0.0332	*CYP2S1*	This gene encodes a member of the cytochrome P450 superfamily of enzymes. CYP2S1 is inducible by dioxin, the induction being mediated by the Aryl Hydrocarbon Receptor (AHR) and Aryl Hydrocarbon Nuclear Translocator (ARNT). The observed ubiquitous tissue distribution, as well as the expression of CYP2S1 throughout embryogenesis suggest that CYP2S1 is likely to metabolize important endogenous substrates; thus far, retinoic acid has been identified.	Cytochrome P450, aryl hydrocarbon signalling	[refseq], [226]
-0.23	0.0332	*NHN1*	conserved nuclear protein NHN1	Unknown	[refseq]
0.96	0.0332	*SLCO2B1*	solute carrier organic anion transporter family, member 2B1. Possible drug transporter.	Solute transporter	[refseq]
1.31	0.0341	*CD14*	CD14 is a surface protein preferentially expressed on monocytes/macrophages, and associates with TLR4 in lipopolysaccaride binding.	Monocytes, macrophages, lipopolysaccharide, pattern recognition receptor	[refseq]
0.2	0.0347	*NAIP*	NOD-like receptor	NOD-like receptor, inflammasome, monocyte, macrophage	[82]
1.09	0.0347	*ITGB2*	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit). This gene encodes the integrin beta chain beta 2. A given chain may combine with multiple partners resulting in different integrins. For example, beta 2 combines with the alpha L chain to form the integrin LFA-1, and combines with the alpha M chain to form the integrin Mac-1. Integrins are known to participate in cell adhesion as well as cell-surface mediated signalling. Defects in this gene are the cause of leukocyte adhesion deficiency type I (LAD1).	Integrins, leukocytes, signalling	[refseq]
1.27	0.0347	*MS4A6A*	This gene encodes a member of the membrane-spanning 4A gene family. Members of this nascent protein family are characterized by common structural features and similar intron/exon splice boundaries and display unique expression patterns among hematopoietic cells and nonlymphoid tissues.	Unknown	[refseq]
-0.75	0.0347	*HOXC4*	This gene belongs to the homeobox family of genes. The homeobox genes encode a highly conserved family of transcription factors that play an important role in morphogenesis in all multicellular organisms.	Homeobox gene, morphogenesis	[refseq]
1.6	0.0347	*HLA-DQA1*	HLA-DQA1 is one of the HLA class II alpha chain paralogues. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells (APC: B lymphocytes, dendritic cells, macrophages).	HLA class II, B cells, dendritic cells, macrophages	[refseq]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
0.39	0.0347	*IQGAP2*	This gene encodes a member of the IQGAP family. The protein contains three IQ domains, one calponin homology domain, one Ras-GAP domain and one WW domain. It interacts with components of the cytoskeleton, with cell adhesion molecules, and with several signaling molecules to regulate cell morphology and motility. Putative tumor suppressor.	Cytoskeleton, cell adhesion, signaling, tumor suppressor	[refseq], [279]
-0.2	0.0347	*MAP2K7*	The protein encoded by this gene is a dual specificity protein kinase that belongs to the MAP kinase kinase family. This kinase is involved in the signal transduction mediating the cell responses to proinflammatory cytokines, and environmental stresses. Expressed by macrophages and involved in osteoclastogenesis.	MAP kinase signal transduction, cytokine response, macrophages, osteoclasts	[refseq],[282]
-0.48	0.0350	*BRWD1*	This gene encodes a member of the WD repeat protein family. Members of this family are involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation. This protein contains 2 bromodomains and multiple WD repeats, and the function of this protein is not known.	Unknown	[refseq]
1.2	0.0355	*HCST*	HCST (or DAP10) is a signaling molecule which associates with C-type lectins, such as CLEC5A.	C-type lectin, pattern recognition signaling, osteoclasts.	[106]
0.91	0.0358	*DOCK2*	Dedicator of cytokinesis 2 (DOCK2) gene encodes a hematopoietic cell-specific CDM family protein that is indispensable for lymphocyte chemotaxis.	Hematopoietic cells, chemotaxis	[refseq]
0.44	0.0358	*ADAM28*	This gene encodes a member of the ADAM (a disintegrin and metalloprotease domain) family. Members of this family are membrane-anchored proteins structurally related to snake venom disintegrins, and have been implicated in a variety of biological processes involving cell-cell and cell-matrix interactions, including fertilization, muscle development, and neurogenesis. The protein encoded by this gene is a lymphocyte-expressed ADAM protein.	Cell-cell interaction, lymphocytes	[refseq]
0.77	0.0361	*LOC387841*	ribosomal protein L13a pseudogene 20	Unknown	[refseq]
1.06	0.0363	*HCLS1*	Hematopoietic cell-specific Lyn substrate 1, actin-regulatory adaptor protein at the immune synapse	Hematopoietic cells, src family kinase substrate, cytoskeleton	[85]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
0.9	0.0371	*SLC11A1*	Natural resistance-associated macrophage protein 1. This gene is a member of the solute carrier family 11 (proton-coupled divalent metal ion transporters) family and encodes a multi-pass membrane protein. The protein functions as a divalent transition metal (iron and manganese) transporter involved in iron metabolism and host resistance to certain pathogens. Mutations in this gene have been associated with susceptibility to infectious diseases such as tuberculosis and leprosy, and inflammatory diseases such as rheumatoid arthritis and Crohn disease.	Solute carrier, macrophages, tuberculosis	[refseq]
-0.08	0.0371	*LOC652140*	similar to DNA-directed RNA polymerase II largest subunit	Unknown	[refseq]
0.45	0.0371	*MARCH1*	Membrane-associated ring finger (C3HC4) 1 is a major regulator of HLA-DR traffic.	HLA class II, B cells, dendritic cells, macrophages	[55]
0.66	0.0374	*SEMA4A*	SEMA4A is a member of the semaphorin family of soluble and transmembrane proteins. Semaphorins are involved in guidance of axonal migration during neuronal development and in immune responses, particularly Th1 type responses through the receptor Tim-2.	Semaphorin, axonal migration, immunology, Th-1 response	[refseq], [187]
0.54	0.0374	*CD86*	This protein is expressed by antigen-presenting cells (APC: B lymphocytes, dendritic cells, macrophages) and it is the ligand for two proteins at the cell surface of T cells, CD28 antigen and cytotoxic T-lymphocyte-associated protein 4. Binding of this protein with CD28 antigen is a costimulatory signal for activation of the T-cell. Binding of this protein with cytotoxic T-lymphocyte-associated protein 4 negatively regulates T-cell activation and diminishes the immune response.	B cells, dendritic cells, macrophages, co-stimulation of T cells	[refseq]
0.92	0.0390	*LST1*	Higher expressed on CD16+ than CD16- monocytes. It is expressed in T cell, monocytic and macrophage cell lines, and it is substantially expressed in both primary human and murine dendritic cells (DCs) in culture.	Monocytes, macrophages, T cells, DCs	[6;189]
0.64	0.0398	*TLR8*	TLR8 recognizes nucleoside analogues. It is expressed in a variety of different cell types of the immune system such as DCs, B cells, monocytes, NK cells and T cells.	Toll-like receptor, pattern recognition receptor, dendritic cells, B cells, monocytes, NK cells, T cells, nucleoside analogues	[16]
0.57	0.0398	*BIN2*	The Bin2 gene is expressed predominantly in hematopoietic cells and is upregulated during differentiation of granulocytes.	Hematopoietic cells, granulocytes	[81]
0.83	0.0398	*TMEM149*	transmembrane protein 149	Unknown	[refseq]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
0.16	0.0398	*MICALCL*	MICAL C-terminal like, function unknown, but MICAL has a role in semaphorin signaling	Unknown, possibly semaphorin signaling	[refseq], [127]
1.29	0.0398	*MS4A6A*	This gene encodes a member of the membrane-spanning 4A gene family. Members of this nascent protein family are characterized by common structural features and similar intron/exon splice boundaries and display unique expression patterns among hematopoietic cells and nonlymphoid tissues.	Unknown	[refseq]
0.56	0.0405	*TLR4*	This receptor is most abundantly expressed in placenta, and in the myelomonocytic subpopulation of the leukocytes. It has been implicated in signal transduction events induced by lipopolysaccharide found in most gram-negative bacteria.	Toll-like receptor, placenta, monocytes, macrophages, lipopolysaccharide, pattern recognition receptor	[refseq]
1.01	0.0405	*GIMAP4*	This gene encodes a protein belonging to the GTP-binding superfamily and to the immuno-associated nucleotide (IAN) subfamily of nucleotide-binding proteins. Upregulated during Th1 differentiation.	GTP-binding protein, Th1 cells	[refseq], [67]
0.4	0.0415	*C12orf35*	chromosome 12 open reading frame 35	Unknown	[refseq]
0.26	0.0415	*CUGBP2*	CUG triplet repeat, RNA binding protein 2, members of this protein family regulate pre-mRNA alternative splicing and may also be involved in mRNA editing, and translation.	RNA binding protein, alternative splicing	[refseq]
0.55	0.0415	*MFNG*	This gene is a member of the fringe gene family which also includes radical and lunatic fringe genes. They all encode evolutionarily conserved secreted proteins that act in the Notch receptor pathway to demarcate boundaries during embryonic development. While their genomic structure is distinct from other glycosyltransferases, fringe proteins have a fucose-specific beta-1,3-N-acetylglucosaminyltransferase activity that leads to elongation of O-linked fucose residues on Notch, which alters Notch signaling.	Notch receptor pathway signaling. Gycosyltransferase.	[refseq]
0.21	0.0415	*NLRC4*	NOD-like receptor	NOD-like receptor, inflammasome, monocyte, macrophage	[82]
-0.29	0.0415	*MRPL4*	Mitochondrial ribosomal protein L4, 39S subunit. Mitochondrial ribosomal proteins are encoded by nuclear genes and help in protein synthesis within the mitochondrion. Mitochondrial ribosomes (mitoribosomes) consist of a small 28S subunit and a large 39S subunit.	Mitochondrial ribosomal protein	[refseq]
0.65	0.0415	*PARVG*	Members of the parvin family, including PARVG, are actin-binding proteins associated with focal contacts. Essential for the establishment of cell polarity required for leukocyte migration.	Cytoskeleton, leukocyte migration	[refseq], [284]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
0.49	0.0415	*AOAH*	Acyloxyacyl hydrolase (AOAH) is a 2-subunit lipase which selectively hydrolyzes the secondary (acyloxyacyl-linked) fatty acyl chains from the lipid A region of bacterial endotoxins. AOAH may modulate host inflammatory responses to gram-negative bacterial invasion. Expressed by leukocytes.	Lipase, bacterial endotoxins, leukocytes.	[refseq]
0.45	0.0415	*PSCD1*	The protein encoded by this gene is a member of the PSCD family. Members of this family appear to mediate the regulation of protein sorting and membrane trafficking. This gene is highly expressed in natural killer and peripheral T cells, and regulates the adhesiveness of integrins at the plasma membrane of lymphocytes.	Natural killer cells, T cells, integrins	[refseq]
0.7	0.0415	*IL10RA*	The protein encoded by this gene is a receptor for interleukin 10. This protein is structurally related to interferon receptors. It has been shown to mediate the immunosuppressive signal of interleukin 10, and thus inhibits the synthesis of proinflammatory cytokines. This receptor is reported to promote survival of progenitor myeloid cells through the insulin receptor substrate-2/PI 3-kinase/AKT pathway. Activation of this receptor leads to tyrosine phosphorylation of JAK1 and TYK2 kinases.	Leukocytes, monocytes, macrophages, cytokines, PI 3-kinase/AKT pathway, immunosuppression	[refseq]
1.15	0.0415	*ALOX5*	This gene encodes a member of the lipoxygenase gene family and plays a dual role in the synthesis of leukotrienes from arachidonic acid. Leukotrienes are arachidonic acid metabolites which have been implicated in various types of inflammatory responses and mediate production of endogenous PPAR-γ-ligands. Mutations in the promoter region of this gene lead to a diminished response to antileukotriene drugs used in the treatment of asthma and may also be associated with atherosclerosis and several cancers. .	Arachidonic acid lipoxygenase, leukotrienes, PPAR- γ ligand production, astma, cancer	[refseq] [267]
0.31	0.0415	*PAOX*	FAD-dependent polyamine oxidase is one of the key enzymes in the catabolism of polyamines spermidine and spermine.	Polyamine oxidase	[111]
0.49	0.0415	*APPL2*	APPL is an effector of the small GTPase Rab5, a key regulator of early steps of endocytosis. In addition, APPL proteins exert their stimulatory effects on beta-catenin/TCF-dependent transcription by decreasing the activity of a Reptin-containing repressive complex.	Endocytosis, beta-catenin/ TCF-dependent transcription	[212]
1.04	0.0415	*FHL2*	Four and a half LIM domains 2, expressed by heart muscle, cancer cells and osteoblasts. FHL2 transcript levels increased threefold during differentiation of mouse bone marrow cells into osteoblasts. Also influences Wnt signalling.	Osteoblasts, Wnt signalling, heart muscle	[115]
0.48	0.0415	*DPEP2*	DPEP2 belongs to the membrane-bound dipeptidase family. These enzymes hydrolyze a variety of dipeptides, including leukotriene D4, the beta-lactam ring of some antibiotics, and cystinyl-bis-glycine (cys-bis-gly) formed during glutathione degradation.	Dipeptidase	[refseq]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
0.87	0.0415	*PTPN6*	Protein tyrosine phosphatase, non-receptor type 6. This PTP is expressed primarily in hematopoietic cells, and functions as an important regulator of multiple signaling pathways in hematopoietic cells. Amongst others, associates with phosphorylated immunoreceptor tyrosine-based activation motif of Fc gamma RIIa to modulate signaling events in myeloid cells.	Hematopoietic cells, Fc receptor signaling, myeloid cells	[refseq] [79]
1.3	0.0415	*C1QA*	Complement component 1, q subcomponent, A chain. Deficiency is associated with lupus erythematosus and glomerulonephritis. Expressed by monocytes and macrophages.	Complement, SLE, monocytes, macrophages	[refseq] [90]
0.3	0.0415	*MPEG1*	Macrophage expressed 1, may share a distant ancestry to perforin.	Macrophages.	[245]
0.94	0.0415	*HAVCR2*	Phagocytic receptor responsible for cross-presentation of dying cell-associated antigens, expressed on macrophages and DCs. Also expressed by Th1 cells.	Phagocytosis, macrophages, DCs, Th1 cells, apoptosis	[192]
1.11	0.0415	*AMICA1*	Junctional adhesion molecule-like, expressed on hematopoietic cells, mainly monocytes and granulocytes	Adhesion, monocyte, granulocyte	[185]
0.71	0.0415	*IFNGR1*	This gene (IFNGR1) encodes the ligand-binding chain (alpha) of the gamma interferon receptor. Human interferon-gamma receptor is a heterodimer of IFNGR1 and IFNGR2.	Interferon gamma receptor	[refseq], [103]
-0.41	0.0421	*NSUN5*	This gene encodes a member of the evolutionarily conserved NOL1/NOP2/Sun domain family. The encoded protein may function as a DNA methyltransferase in the nucleus.	DNA methyltransferase	[refseq]
-0.08	0.0429	*DNAI2*	Dynein, axonemal, intermediate chain 2 is essential for outer dynein arm assembly. DNAI2 mutations result in primary ciliary dyskinesia and randomization of left/right body asymmetry.	Dynein, cytoskeleton, ciliary dyskinesia	[149]
0.1	0.0434		ns42b07.s1 NCI_CGAP_GCB1 Homo sapiens cDNA clone IMAGE:1186261 3, mRNA sequence	Unknown	
0.41	0.0438	*VAV1*	The protein encoded by this proto-oncogene is a member of the Dbl family of guanine nucleotide exchange factors (GEF) for the Rho family of GTP binding proteins. The protein is important in hematopoiesis, playing a role in T-cell and B-cell development and activation. This particular GEF has been identified as the specific binding partner of Nef proteins from HIV-1. Coexpression and binding of these partners initiates profound morphological changes, cytoskeletal rearrangements and the JNK/SAPK signaling cascade, leading to increased levels of viral transcription and replication.	Oncogene, hematopoietic cells, JNK/SAPK signaling	[refseq]
0.55	0.0438	*MERTK*	C-mer proto-oncogene tyrosine kinase is a receptor involved in phagocytosis of (pro-) apoptotic cells by macrophages. Mutations in this gene have been associated with disruption of the retinal pigment epithelium (RPE) phagocytosis pathway and onset of autosomal recessive retinitis pigmentosa.	Macrophages, apoptosis, phagocytosis, retinitis pigmentosa	[refseq] [265]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
1	0.0438	*LY86*	Myeloid differentiation-1 associates with the toll-like protein RP105 in B cells and is downregulated on monocytes following BCG infection.	Toll signaling, B cells, monocytes	[17;180]
0.61	0.0438	*MYO1G*	Myosin IG, also known as minor histocompatibility antigen HA-2, expressed by hematopoietic cells.	Hematopoietic cells, minor histocompatibility antigen	[197]
0.19	0.0443	*RPGRIP1*	This gene encodes a photoreceptor protein that interacts with retinitis pigmentosa GTPase regulator protein and is a key component of cone and rod photoreceptor cells.	Photoreceptor protein, autosomal recessive congenital blindness	[refseq]
0.42	0.0446	*ASGR1*	The asialoglycoprotein receptor binds to desialylated (galactosyl-terminal) plasma glycoproteins, removing them from the circulation. It transports these glycoproteins via a series of membrane vesicles and tubules to an acidic-sorting organelle where the receptor and ligand dissociate. Then the receptor is recycled back to the cell surface and the ligand is transported to the lysosomes for degradation. Expressed by hepatocytes and macrophages.	Glycoproteins, hepatic cells, endocytosis, macrophages	[refseq], [241]
0.33	0.0446	*PLEKHA7*	Part of the protein-complex in the zonula adherens, a specialized cadherin-based structure found at the contacts between epithelial cells where microtubules attach.	Zonula adherens, cytoskeleton.	[166]
0.83	0.0446	*IL1B*	This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE).	Caspase activation, inflammasome, cytokine, inflammation, osteoclast	[refseq]
0.07	0.0455		AB076959 Human vestibular cDNA library Homo sapiens cDNA clone 415V5-6-11, mRNA sequence	Unknown	
0.06	0.0463	*C20orf174*		Unknown	
-0.06	0.0469	*LOC649978*		Unknown	
-0.95	0.0473	*GHR*	This gene encodes a protein that is a transmembrane receptor for growth hormone. Binding of growth hormone to the receptor leads to receptor dimerization and the activation of an intra- and intercellular signal transduction pathway leading to growth.	Growth hormone receptor	[refseq]
-0.15	0.0473		UI-H-BI1-aem-a-04-0-UI.s1 NCI_CGAP_Sub3 Homo sapiens cDNA clone IMAGE:2719758 3, mRNA sequence	Unknown	
0.66	0.0475	*P2RY13*	The product of this gene belongs to the family of G-protein coupled receptors. This receptor is activated by ADP and is a high-affinity receptor for HDL apolipoprotein A-I (apoA-I) on human hepatocytes.	G-protein coupled receptor, HDL apolipoprotein, human hepatocytes.	[refseq], [108]

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
1	0.0475	*CECR1*	This gene encodes a member of a subfamily of the adenosine deaminase protein family. The encoded protein may act as a growth factor and have adenosine deaminase activity. It may be active in sites of inflammation during hypoxia and in areas of tumour growth.	Adenosine deaminase, inflammation, hypoxia, tumor growth	[refseq], [285]
1	0.0475	*C1orf162*		Unknown	
0.89	0.0475	*PYCARD*	The PYD and CARD domains are members of the six-helix bundle death domain-fold superfamily that mediates assembly of large signaling complexes in the inflammatory and apoptotic signaling pathways via the activation of caspase.	Caspase activation, inflammasome, monocyte, macrophage	[refseq], [74]
0.54	0.0483	*TMBIM4*	Transmembrane BAX inhibitor motif containing 4, may participate in cell death regulation by interacting with proteins of Bcl-2 family, thus promoting tumor metastasis.	BAX, BCL-2, apoptosis, metastasis	[286]
0.36	0.0485	*SLC4A7*	Solute carrier family 4, sodium bicarbonate cotransporter, member 7. Involved in bicarbonate transport, expressed by osteoclasts, heart, skeletal muscle, and kidney in which it plays an important role in HCO(3)(-) metabolism.	Bicarbonate transporter, osteoclasts, heart, msucle, kidney.	[150;215]
0.49	0.0485	*NECAP2*	This gene likely encodes a member of the adaptin-ear-binding coat-associated protein family. Studies of a similar protein in rat suggest a role in clathrin-mediated endocytosis.	Endocytosis	[refseq]
0.91	0.0499	*WAS*	The Wiskott-Aldrich syndrome (WAS) family of proteins share similar domain structure, and are involved in transduction of signals from receptors on the cell surface to the actin cytoskeleton. The presence of a number of different motifs suggests that they are regulated by a number of different stimuli, and interact with multiple proteins. Recent studies have demonstrated that these proteins, directly or indirectly, associate with the small GTPase, Cdc42, known to regulate formation of actin filaments, and the cytoskeletal organizing complex, Arp2/3. The WAS gene product is a cytoplasmic protein, expressed exclusively in hematopoietic cells.	Arp2/3, cytoskeleton, hematopoietic cells	[refseq]
0.45	0.0499	*AADACL1*	Arylacetamide deacetylase-like 1, also known as neutral cholesterol ester hydrolase 1 is expressed by macrophages in atherosclerotic plaques. Also highly expressed in aggressive cancer cells.	Cholesterol ester hydrolase, lipid metabolism, macrophages, cancer	[40;196]
0.08	0.0499		BX112750 Soares placenta Nb2HP Homo sapiens cDNA clone IMAGp998K15220, mRNA sequence	Unknown	
0.07	0.0499		ze37g04.r1 Soares retina N2b4HR Homo sapiens cDNA clone IMAGE:361206 5, mRNA sequence	Unknown	

Suppl. Table 4.4 *Continued*

logFC	adjPVal	Symbol	Description	Keywords	References
-0.27	0.0499	*BCCIP*	BRCA2 and CDKN1A interacting protein, This gene product was isolated on the basis of its interaction with BRCA2 and p21 proteins. It is an evolutionarily conserved nuclear protein with multiple interacting domains. The N-terminal half shares moderate homology with regions of calmodulin and M-calpain, suggesting that it may also bind calcium. Functional studies indicate that this protein may be an important cofactor for BRCA2 in tumor suppression, and a modulator of CDK2 kinase activity via p21. This protein has also been implicated in the regulation of BRCA2 and RAD51 nuclear focus formation, double-strand break-induced homologous recombination, and cell cycle progression. BCCIP is essential for completion of cytokinesis.	BRCA2, p21, DNA damage response pathway, homologous recombination, cell cycle control, cytokinesis.	[refseq], [167]
-0.06	0.0499	*C3orf42*		Unknown	
0.65	0.0499	*LST1*	Higher expressed on CD16+ than CD16- monocytes. It is expressed in T cell, monocytic and macrophage cell lines, and it is substantially expressed in both primary human and murine dendritic cells (DCs) in culture.	Monocytes, macrophages, T cells, DCs	[6;189]
1.32	0.0499	*HLA-DQB1*	HLA-DQB1 belongs to the HLA class II beta chain paralogues. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells (APC: B lymphocytes, dendritic cells, macrophages).	HLA class II, B cells, dendritic cells, macrophages	[refseq]
-0.11	0.0499	*SLC24A4*	Potassium-dependent sodium/calcium exchangers, such as NCKX4, are thought to transport 1 intracellular calcium and 1 potassium ion in exchange for 4 extracellular sodium ions.	Sodium/potassium/calcium exchanger	[refseq]
0.09	0.0499	*GGN*	This gene is a germ cell-specific gene that encodes proteins that interact with POG (proliferation of germ cells). These proteins regulate the localization of POG and may play a role in spermatogenesis.	Germ cell-specific gene, spermatogenesis.	[refseq]

5.

Chemotherapy resistant osteosarcoma is highly susceptible to IL-15 activated allogeneic and autologous NK cells

Emilie P. Buddingh, Marco W. Schilham, S. Eriaty N. Ruslan, Dagmar Berghuis, Karoly Szuhai, Jolien Suurmond, Antonie H.M. Taminiau, Hans Gelderblom, R. Maarten Egeler, Massimo Serra, Pancras C.W. Hogendoorn, Arjan C. Lankester

Cancer Immunol Immunother. 2011 Apr;60(4):575-86.

ABSTRACT

INTRODUCTION: High-grade osteosarcoma occurs predominantly in adolescents and young adults and has an overall survival-rate of about 60%, despite chemotherapy and surgery. Therefore, novel treatment modalities are needed to prevent or treat recurrent disease. Natural killer (NK) cells are lymphocytes with cytotoxic activity towards virus infected or malignant cells. We explored the feasibility of autologous and allogeneic NK cell mediated therapies for chemotherapy resistant and sensitive high-grade osteosarcoma. METHODS: The expression by osteosarcoma cells of ligands for activating NK cell receptors was studied *in vitro* and *in vivo* and their contribution to NK cell mediated cytolysis was studied by specific antibody blockade. RESULTS: Chromium release cytotoxicity assays revealed chemotherapy sensitive and resistant osteosarcoma cell lines and osteosarcoma primary cultures to be sensitive to NK cell mediated cytolysis. Cytolytic activity was strongly enhanced by IL-15 activation and was dependent on DNAM-1 and NKG2D pathways. Autologous and allogeneic activated NK cells lysed osteosarcoma primary cultures equally well. Osteosarcoma patient derived NK cells were functionally and phenotypically unimpaired. CONCLUSION: In conclusion, osteosarcoma cells, including chemoresistant variants, are highly susceptible to lysis by IL-15-induced NK cells from both allogeneic and autologous origin. Our data support the exploitation of NK cells or NK cell activating agents in patients with high-grade osteosarcoma.

INTRODUCTION

High-grade osteosarcoma is the most common primary malignant bone sarcoma, occurring mainly in adolescents and young adults [125]. Despite multi-agent chemotherapy and surgery, overall survival is still poor at about sixty percent [24;32;141]. Therefore, novel treatment modalities are urgently needed to either prevent or treat chemotherapy refractory and recurrent disease. Immunomodulatory agents such as interferon (IFN)-α and muramyl-tripeptide (MTP) have been added to standard chemotherapy regimens in recent clinical trials [160;169;170]. Immunotherapy with stimulatory cytokines such as IL-2 and IL-15, or the adoptive transfer of *ex vivo* cytokine activated cytotoxic lymphocytes such as natural killer (NK) cells could be another adjunct to current therapy [148;174;255].

NK cells lack a clonally rearranged antigen-specific receptor. Instead, cytolytic activity towards virus infected or malignant cells is dependent on the balance between inhibitory and activating signals. NK cell activating signals are provided when the activating receptors Natural Killer Group 2, member D (NKG2D), DNAX accessory molecule-1 (DNAM-1) and the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 bind their respective ligands [178;186;272]. Although NK cell recognition of tumor cells has been reported to be partially mediated through NCRs, the responsible ligands are unknown [135]. The DNAM-1 ligands poliovirus receptor (PVR, CD155) and Nectin-2 (CD112) are highly expressed by many tumors, including sarcomas [26;64;272]. Ligands for NKG2D are the stress-inducible major histocompatibility class I polypeptide-related sequence (MIC) A and B and the UL-16 binding proteins (ULBPs) 1 to 4. Inhibitory ligands are the classical and non-classical human leukocyte antigen (HLA) class I molecules expressed on all normal cells [118]. These ligands bind to inhibitory killer immunoglobulin-like receptors (KIRs) and the C-type lectin heterodimer CD94/NKG2A on NK cells, respectively. In addition to high expression of NKG2D and DNAM-1 ligands, many tumors show loss of HLA class I, possibly rendering them susceptible to NK cell mediated lysis [20;38].

In the current study, we demonstrated the sensitivity of chemotherapy resistant and sensitive osteosarcoma cells to lysis by IL-15 activated NK cells and identified the molecular mechanisms involved. NK cells of osteosarcoma patients were not functionally impaired and were able to lyse autologous tumor cells, supporting the use of NK cell activating agents in the treatment of osteosarcoma patients.

MATERIALS AND METHODS

Patient material

A tissue array was constructed from formalin fixed, paraffin embedded (FFPE) tissue retrospectively collected from 88 osteosarcoma patients treated at the LUMC as previously described [183] (Suppl. Table 5.1). Peripheral blood mononuclear cells (PBMCs) were collected from healthy controls and 22 pre-treatment osteosarcoma patients after written informed consent was obtained, as approved by the Institutional Review Board on Medical Ethics. Osteosarcoma tissue samples were used for research in accordance with national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of

Table 5.1 Expression of inhibitory and activating NK ligands by osteosarcoma. Eight osteosarcoma cell lines (HOS, 143B/HOS, SJSA-1, OHS, ZK-58, U2-OS and SAOS-2), six chemotherapy resistant variant cell lines (doxorubicin (DX), methotrexate (MTX) and cisplatinum (CDDP) resistant variants of U2-OS and SAOS-2) and five short-term cultures (L2808, L2599, L2792, L2635 and L2531; all no later than passage 3) were evaluated for the expression of NK cell ligands by flow cytometry. Expression of the inhibitory ligands CD48 and MHC class I and expression of ligands for the activating receptors NKG2D (MICA, MICB, ULBP-1, ULBP-2 and ULBP-3) and DNAM-1 (CD112 and CD155). Expression of the adhesion molecules CD54 and CD58 and of the death receptor CD95 (Fas). (−) mean fluorescence intensity (MFI) ratio of specific staining versus isotype control <2; (+/−) MFI ratio between 2 and 5; (+) MFI ratio between 5 and 10; (++) MFI ratio >10.

							U2-OS				SAOS-2								
	HOS	143B/ HOS	SJSA-1	IOR/ OS-14	OHS	ZK-58	U2-OS	DX 580	MTX 300	CDDP 4	SAOS-2	DX 580	MTX 1	CDDP 6	L2531	L2599	L2635	L2792	L2808
MHC class I	++	++	++	++	+/-	+	++	++	++	++	++	++	++	++	++	++	++	++	++
CD48 (2B4 ligand)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MICA	+/-	+	+/-	+/-	-	+/-	++	+	+	++	++	++	++	+	-	-	++	+/-	-
MICB	-	+	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ULBP-1	+/-	+	+/-	+/-	+/-	-	-	-	-	-	-	+/-	+/-	+/-	-	-	-	-	-
ULBP-2	+/-	++	+	+	+	+	++	+	++	+/-	++	++	++	++	-	+/-	+/-	+/-	+/-
ULBP-3	-	+/-	-	+/-	+	+/-	+	+/-	+	+/-	+/-	-	+/-	-	-	-	-	+/-	+/-
CD112 (Nectin-2)	+	+	++	+	+/-	+/-	++	++	++	++	+	+/-	+	+/-	+/-	+/-	+/-	+/-	++
CD155 (PVR)	+/-	++	+	+	+	+/-	++	++	++	+	+	+	+	+	+/-	+/-	++	+/-	+
CD54 (ICAM-1)	++	++	++	++	-	+/-	-	-	-	-	-	-	-	-	-	++	+/-	+/-	+
CD58 (LFA-3)	++	+	++	+	++	++	++	+	++	++	++	++	++	+	+	+/-	++	+/-	+
CD95 (Fas)	+/-	+/-	+/-	+/-	+/-	+/-	+	+/-	+	+	+/-	-	+/-	-	+/-	+	-	+/-	+

Medical Scientific Societies). All patient material was handled in a coded fashion. Clinical and pathological details of all patients can be found in Suppl. Table 5.2.

Cell lines and primary cultures

The osteosarcoma cell lines HOS, 143B/HOS, IOR-OS14, SJSA-1, OHS, ZK-58, U2-OS and SAOS-2 were characterized and maintained as described earlier [199]. The EBV B-LCL cell line 107 (EBV) and the erythroleukemic cell line K562 were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Invitrogen) and 1% penicillin/streptomycin (PS, Invitrogen). The chemotherapy resistant variant cell lines of U2-OS and SAOS-2 were established as described previously [204;235;236] and were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with 10% FCS and PS. The doxorubicin (DX) resistant variants U2-OS-DX580 and SAOS-2-DX580 were cultured in the presence of 580 µg/mL DX. The methotrexate (MTX) resistant variants U2-OS-MTX300 and SAOS-2-MTX1µg were cultured in the presence of 300 and 1000 ng/mL MTX, respectively. The cisplatin (cis-diamminedichloroplatinum, CDDP) resistant variants U2-OS-CDDP4µg and SAOS-2CDDP6µg were cultured in the presence of 4 and 6 µg/mL CDDP respectively.

Fresh osteosarcoma samples L2808, L2599, L2792, L2635 and L2531 were cultured as described previously by our group for related tumors [252]. Clinical and histopathological details can be found in Suppl. Table 5.3. L2531, L2792 and L2599 were derived from patients with poor histological response to pre-operative chemotherapy in the primary tumor. L2808, a pulmonary metastatic sample, was derived from a patient with good histological response in the primary tumor but who relapsed nonetheless. L2635 originated from a patient with good histological response to pre-operative chemotherapy who is currently in persistent first complete remission (follow-up since diagnosis 20 months). Collected tissue pieces were dissociated mechanically and cultured in RPMI 1640 medium supplemented with 20% FCS and PS. When subconfluence was reached, cells were harvested using 0.05% Trypsin/EDTA (Invitrogen) and passaged. Chromium release assays and flow cytometric analyses were performed no later than at passage 3.

Isolation and culture of NK cells

PBMCs were isolated using a Ficoll density gradient separation followed by NK cell enrichment using the MACS NK enrichment kit and LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Purity of NK cells was assessed by flow cytometry and was typically around 95% (less than 1% T cells). NK cells were cultured in AIM-V medium (Invitrogen), supplemented with 10% pooled human AB serum (Sanquin, Rotterdam, Netherlands), PS and glutamine (Glutamax I, Invitrogen). Activated NK cells were cultured with 10 ng/mL recombinant human interleukin-15 (IL-15) for three days or two weeks (Peprotech, Rocky Hill, NJ).

Flow cytometry

Surface staining of cells for flow cytometry was performed as described elsewhere [20]. Intracellular flow cytometry staining was done using permeabilization and fixation kits as per manufacturer's instructions (00-5123; 00-5223; 00-8333, eBioscience, San Diego, CA). An overview of antibodies can be found in Suppl. Table 5.4. Flow cytometry of PBMC of osteosarcoma patients

and healthy controls was performed on a BD LSRII and analyzed using FACS Diva Software 5.0 (both from Becton Dickinson, San Diego, CA). Mean fluorescence intensity (MFI) of cell subsets was calculated by subtracting the MFI of a negative population from the MFI of the population of interest within one individual to correct for interindividual variability of background staining. Flow cytometry of cell lines and purified NK cells was performed on a FACScalibur, and analyzed using Cellquest software (both Becton Dickinson). MFI ratio was calculated by MFI of the specific staining relative to the MFI of the appropriate isotype control staining.

Immunohistochemistry

Immunohistochemistry was performed on FFPE tissue array sections as previously described [183]. Testis was used as a positive control for the activating NK ligands and tonsil for the inhibitory ligands. Sections were blocked using 10% swine or goat serum in PBS and subsequently incubated with primary antibody diluted in 0.5% bovine serum albumin (BSA) in PBS overnight. As a negative control 0.5% BSA/PBS without primary antibody was used. All primary antibodies are listed in Suppl. Table 5.4. Anti-rabbit/rat/mouse PowerVision Poly-HRP (Leica Biosystems, Newcastle Upon Tyne, United Kingdom) was used as a secondary antibody, except for the MICA staining, in which case the Universal LSAB+ Kit (DAKO, Glostrup, Denmark) was used. DAB+ (DAKO) was used as a chromogen. Sections were counterstained using Mayer's hematoxylin. Tissue array images were acquired using the MIRAX slide scanner (3DHISTECH, Budapest, Hungary) and analyzed using the MIRAX viewer version 1.14 (3DHISTECH). Slides were scored by two observers (EPB and PCWH) in a modified semi-quantitative scoring system as proposed by Ruiter *et al* [225]. The intensity of staining was scored as 0, 1, 2 or 3 indicating absent, weak, clear or strong expression, respectively. Percentages of positive cells were scored as 0 for 0%, 1 for 1-30%, 2 for 31-70% and 3 for 71-100%.

Chromium release assays

Cytotoxicity was determined in standard four hour Chromium release assays. For experiments using PBMCs of OS patients and controls, PBMCs were thawed from storage in liquid nitrogen and allowed to recover for 16 h in RPMI 1640 supplemented with 10% FCS and PS. The E:T ratios in these experiments were corrected for the percentage of NK cells of PBMCs as determined by flow cytometry. For all other experiments, purified unstimulated or IL-15 activated NK cells were used as effector cells. Target cells (cell lines or primary cultures) were incubated with 100 µCi sodium-51-chromate (PerkinElmer, Wellesley, MA) for 1 h. Effector cells (PBMCs, unstimulated purified NK cells or activated NK cells) were incubated for 4 h with 2500 target cells at eight effector:target (E:T) ratios in triplicate. Maximum and spontaneous release was determined by incubating targets in 2N HCl or medium, respectively. Supernatants were harvested and measured in a gamma-counter (Wallac, PerkinElmer). Specific lysis was determined as: (experimental release-spontaneous release)/(maximum release-spontaneous release) × 100%. In all NK cytotoxicity experiments, K562 and EBV were used as positive and negative controls, respectively. For blocking experiments, NK cells preincubated with blocking anti-NKG2D (R&D systems, clone 149810) and/or blocking anti-DNAM-1 (BD Pharmingen, clone DX11) at a concentration of 20 µg/mL. To disrupt perforin/granzyme mediated cytolysis, NK cells were preincubated for 2 h at 37°C with or without

1 μM Concanamycin A (Sigma-Aldrich, Zwijndrecht, the Netherlands) prior to adding the NK cells to the target cells. To block Fas-induced apoptosis, target cells were preincubated with 2μg/mL neutralizing anti-Fas antibody (Clone ZB4, Millipore, Temecula, CA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (LaJolla, CA). Data with non-normal distribution or small sample size were analyzed using non-parametric methods (Mann-Whitney U, Kruskal-Wallis, Friedman and Dunns tests) and data with normal distribution were analyzed using parametric methods (t-tests, one way analysis of variance (ANOVA) and Bonferroni's tests). Survival analyses were performed using Kaplan-Meier curves and compared using the logrank method.

RESULTS

Osteosarcoma cells are highly susceptible to IL-15 activated allogeneic NK cells

We tested eight osteosarcoma cell lines for susceptibility to cytolytic activity of freshly isolated ('unstimulated') and IL-15 cultured ('activated') healthy donor derived NK cells. All cell lines were lysed by unstimulated allogeneic NK cells at levels comparable to the positive control cell line K562 (Fig. 5.1a and b). Cytolysis of all osteosarcoma cell lines was strongly enhanced when IL-15 cultured allogeneic NK cells were used.

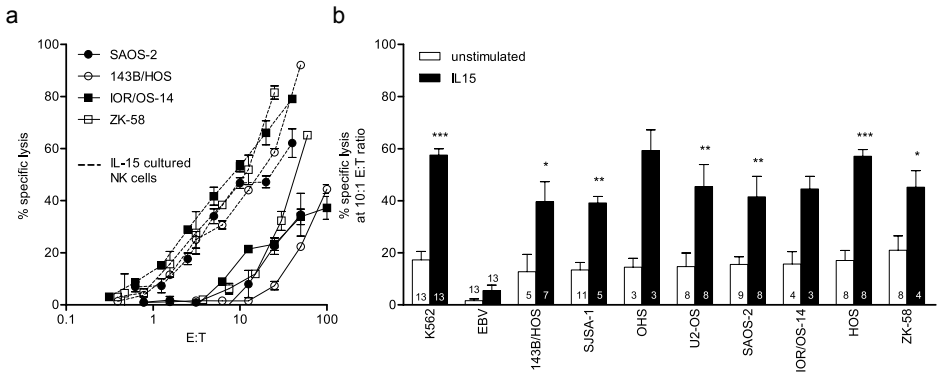


Fig. 5.1 Osteosarcoma cells were sensitive to lysis by freshly isolated NK cells (solid lines) and NK cells cultured in IL-15 for 2 weeks (dashed lines). **a**, examples of percentage of specific lysis are shown for the osteosarcoma cell lines SAOS-2 (●), 143B/HOS (○), IOR/OS-14 (■) and ZK-58 (□). Cell lines were incubated with increasing numbers of NK cells (E:T; effector to target ratio). Error bars represent standard error of the mean lysis of a representative experiment performed in triplicate. **b**, mean percentage of specific lysis by unstimulated (white bars) and IL-15 activated (black bars) NK cells of the osteosarcoma cell lines 143B/HOS, SJSA-1, OHS, U2-OS, SAOS-2, IOR/OS-14, HOS and ZK-58 at an effector to target ratio of 10:1. Error bars represent standard error of the mean of independent experiments using different healthy donor NK cells. Numbers in the bars represent number of experiments. K562 and an EBV transformed B-LCL ("EBV") were used as positive and negative controls, respectively. Mann-Whitney-U test was done comparing IL-15 activated NK cells with unstimulated NK cells for each cell line; *P*-value <0.05 noted as *; <0.01 = **; <0.001 = ***

Osteosarcoma cells express inhibitory and activating NK cell ligands

Osteosarcoma cells expressed activating NK cell ligands and HLA class I, both *in vivo* and *in vitro* (Table 5.1 and Fig. 5.2). All osteosarcoma cell lines expressed HLA class I, at least 3/5 NKG2D ligands and both DNAM-1 ligands. Expression of ligands *in vivo* was determined on the tissue array containing 144 samples of 88 patients. In chemotherapy-naive tumor material MICA, DNAM-1 ligands and HLA class I were also expressed, albeit at different levels (Fig. 5.2a). In tumor cells persisting after chemotherapy, levels of MICA, HLA class I and β-2 microglobulin expression were unaltered but the expression levels of the DNAM-1 ligands CD112 and CD155 were significantly decreased (Fig. 5.2b). There was a trend for patients with high (score > 4) vs. low (score </= 4) expression of MICA in pre-treatment diagnostic biopsies to have better overall survival (n=53, *P*-value logrank test = 0.07). Expression level of HLA class I in diagnostic biopsies as determined by staining with antibodies recognizing β-2 microglobulin, HLA-A and HLA-B/C did not correlate with tumor progression.

NK cells lyse osteosarcoma cells in a DNAM-1 and NKG2D dependent manner

NK cell-mediated cytolysis of osteosarcoma cells was dependent on NKG2D and DNAM-1 pathways, as was demonstrated by blocking DNAM-1, NKG2D or both receptors (Fig. 5.3). In resting NK cells the DNAM-1 pathway appeared to predominate the cytolytic potential, whereas the contribution of the NKG2D pathway was more prominent in the cytolytic activity of IL-15 cultured NK cells. In case of IL-15 cultured NK cells, blockade of both pathways was required for optimal inhibition of NK cytolytic activity. Levels of expression of HLA class I did not correlate with magnitude of lysis by unstimulated or IL-15 activated NK cells. Similarly, levels of expression of ligands for the activating receptors NKG2D or DNAM-1 did not correlate with degree of lysis by NK cells (data not shown).

Chemotherapy resistant osteosarcoma cells remain sensitive to lysis by IL-15 activated NK cells

To study whether chemotherapy resistant cell lines have become resistant to NK cell mediated lysis as well, the sensitivity of a panel of chemotherapy resistant variants of the osteosarcoma cell lines SAOS-2 and U2-OS (selected *in vitro* to be resistant to DX, CDDP or MTX) to lysis by NK cells was tested (Fig. 5.4a). Although some SAOS variants, *e.g.* CDDP, were less sensitive to lysis by resting NK cells, activation of NK cells with IL-15 greatly enhanced lysis of all U2-OS and SAOS-2 chemotherapy resistant variant cell lines (Fig. 5.4a). Expression levels of NKG2D and DNAM-1 ligands were similar in chemotherapy resistant variants and parental cell lines, as was the dependency on NKG2D and DNAM-1 signaling in cytotoxicity assays (Table 5.1 and Suppl. Fig. 5.1). Expression levels of HLA class I and of the adhesion molecules ICAM-1 and LFA-3 were unaltered in the chemotherapy resistant variants, but expression of CD95 (death receptor Fas) was lost in the SAOS-2 CDDP and DX-resistant variants (Table 5.1 and Fig. 5.4b). Since the loss of CD95 could provide an explanation for reduced susceptibility to NK cell induced lysis, we performed blocking experiments in which both CD95 and the granule exocytosis pathway were blocked with a blocking antibody and Concanamycin A, respectively. These experiments were performed using IL-15 activated NK cells at an effector to target ratio of 40 to 1. Blocking the

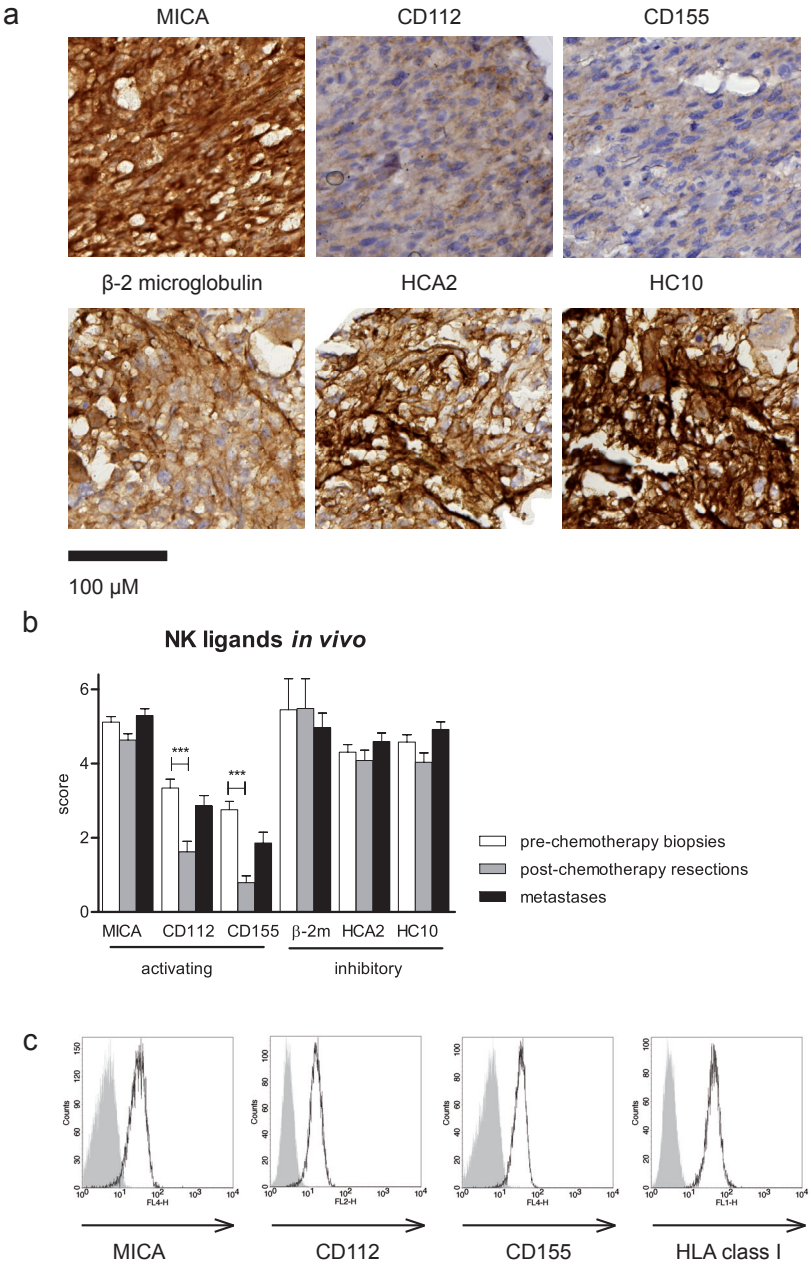


Fig. 5.2 **a**, representative example of immunohistochemical staining of ligands for the activating receptors NKG2D (MICA) and DNAM-1 (CD112 and CD155) and of β2-microglobulin, HLA-A (HCA2) and HLA-B/C (HC10) on an osteosarcoma sample. **b**, overview of the results of immunohistochemical stainings on pre-chemotherapy and post-chemotherapy samples of the primary tumor as well as metastatic osteosarcoma tissue. Expression levels of CD112 and CD155 but not the other ligands decreased significantly upon chemotherapy treatment (*P*-value Kruskal-Wallis test <0.001=***). **c**, example of flow cytometry plots for MICA, CD112, CD155 and HLA class I for the osteosarcoma cell line IOR/OS-14; isotype matched control staining is shown in grey

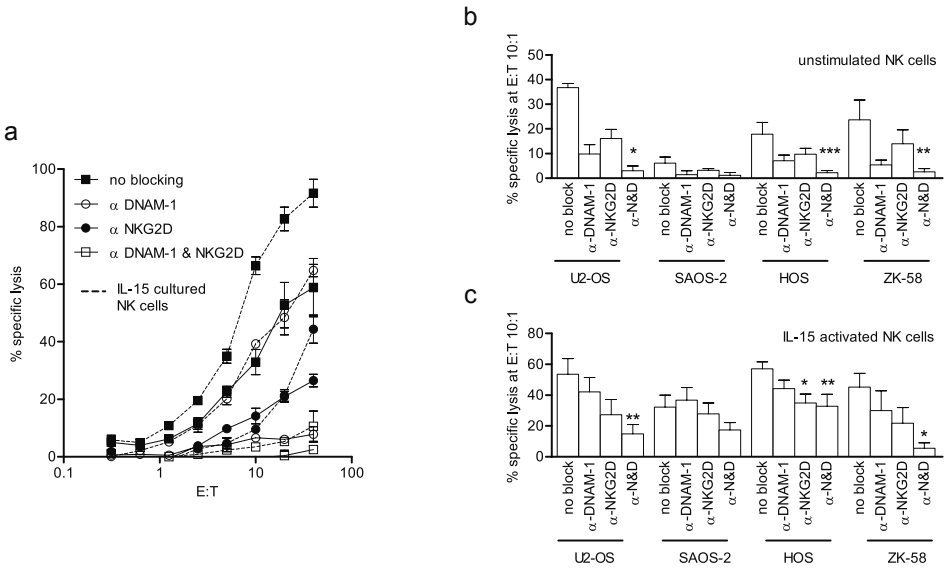


Fig. 5.3 **a**, cytolysis of U2-OS by unstimulated (solid lines) and IL-15 activated (dashed lines) NK cells was almost completely abrogated when the NK cells were pre-incubated with both anti (α)-DNAM-1 and α-NKG2D blocking antibodies (■ *vs.* □). Unstimulated NK cells were most dependent on DNAM-1 (○) signaling, whereas activated NK cells were most dependent on NKG2D (●). Error bars represent standard error of the mean lysis of experiment performed in triplicate. Similar results were obtained for SAOS-2, HOS and ZK-58 using unstimulated (**b**) and IL-15 activated NK cells (**c**). Bars represent mean lysis in at least three independent experiments using healthy donor NK cells; error bars represent standard error of the mean. Friedman test, Dunns post test compared to non-blocked; *P*-value <0.05 noted as *; <0.01 = **; <0.001 = ***

GrB but not the CD95 cytolytic pathway, almost completely abrogated NK cytolytic potential, demonstrating the predominance of the GranzymeB pathway in NK cell mediated lysis of parental as well as CDDP resistant variants of osteosarcoma cells (Fig. 5.4c). Similar results were obtained when the U2-OS parental cell line was used (data not shown).

Peripheral blood NK cell phenotype is unaltered and cytolytic potential is unimpaired in newly diagnosed osteosarcoma patients

Since peripheral NK cells in patients with other tumor types show altered phenotype and function, we analyzed PBMCs of 22 newly diagnosed osteosarcoma patients and 23 age-matched healthy controls by flow cytometry for NK cell number and phenotype (Suppl. Fig. 5.2a). NK cell number and phenotype were comparable between patients and controls (Fig. 5.5a and Suppl. Fig. 5.2b and c). Following three days of culture in IL-15, there was a larger increase in NKG2D and granzyme B expression levels on both CD56dim and bright NK cells of osteosarcoma patients than of healthy controls (Fig. 5.5a and Suppl. Fig. 5.2c). We assessed the functionality of NK cells of osteosarcoma patients at diagnosis in cytotoxicity assays using unstimulated and 3 days IL-15 activated PBMCs as effector cells. Resting NK cells from osteosarcoma patients and healthy donors lysed the allogeneic target HOS equally well (Fig. 5.5b), but IL-15 cultured NK cells of patients lysed HOS significantly

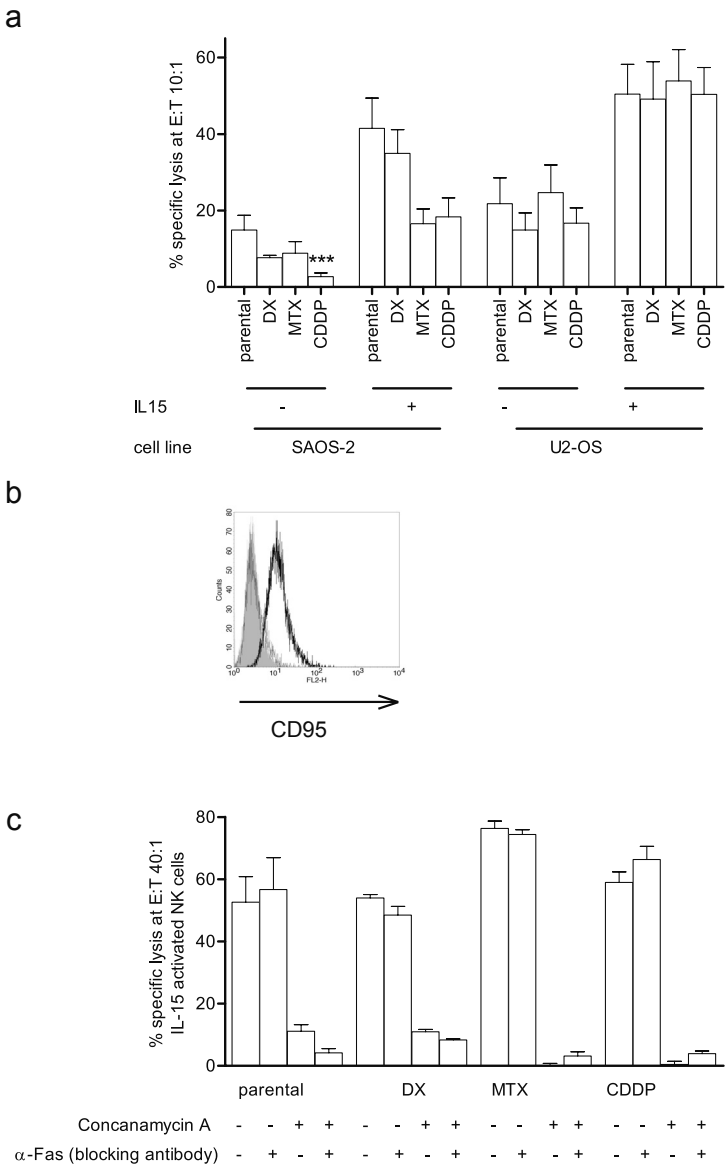


Fig. 5.4 **a**, lysis of U2-OS variants resistant to doxorubicin (DX), methotrexate (MTX) or cisplatinum (CDDP) was comparable to lysis of the parental control. The SAOS-2 CDDP resistant line was less sensitive to lysis by unstimulated NK cells than its parental control (Friedman test *P*-value = 0.001 and Dunns post test compared to parental cell line *P*-value <0.001). NK cell activation ("IL-15 +") increased lysis in all cases. Error bars represent standard error of the mean lysis in at least six independent experiments. **b**, SAOS-2 expressed the death receptor CD95 (Fas; black line) whereas expression was lost in the cisplatinum resistant variant of SAOS-2 (dashed line). Isotype matched control staining is shown in grey. **c**, the granzyme/perforin pathway was the main cytotoxic pathway by which NK cells lysed SAOS-2 and its chemotherapy resistant variants, as shown by inhibition of granule exocytosis by concanamycin A. Blocking Fas resulted in a further decrease in lysis of SAOS-2, but not of the chemotherapy resistant variants. Bars represent mean lysis of experiment performed in triplicate; error bars represent standard error of the mean. Similar results were obtained using NK cells from another donor

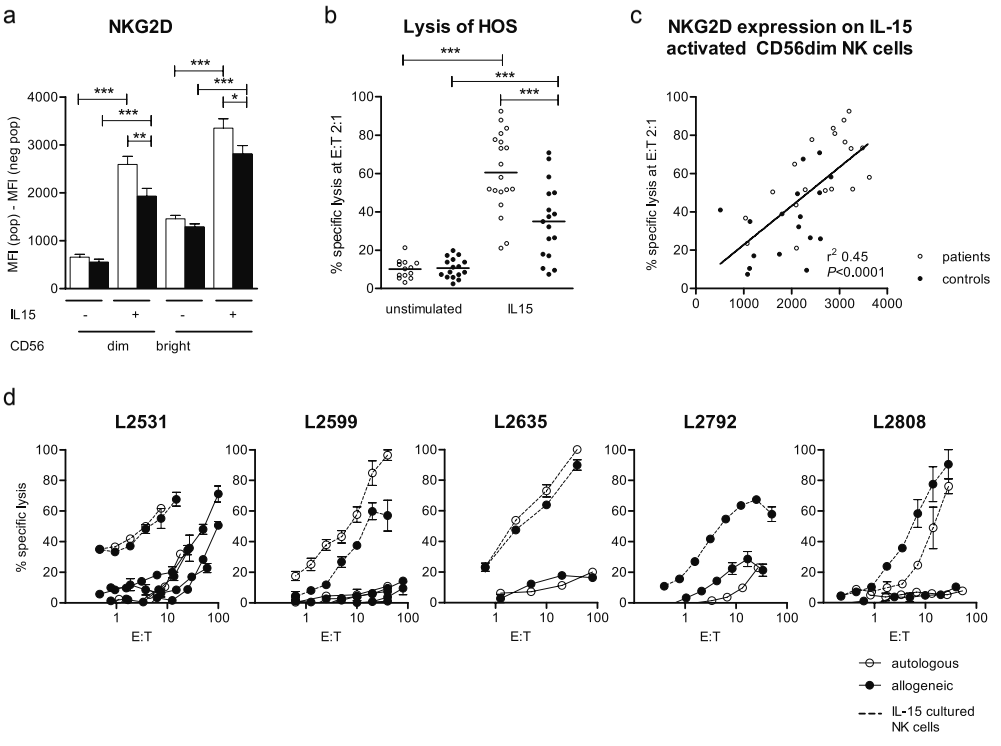


Fig. 5.5 **a**, NKG2D level was similar in unstimulated NK cells of newly diagnosed osteosarcoma patients and healthy controls. Following culture for 3 days in IL15, there was a larger increase in expression level of NKG2Din NK cells of patients. **b**, unstimulated PBMCs of 12/22 patients and 16/23 healthy controls and IL-15 activated PBMCs of 19/22 patients and 17/23 controls were available for functional testing. NK cells of newly diagnosed osteosarcoma patients and of healthy controls lysed HOS at similar levels. Following IL-15 activation, NK cells of osteosarcoma patients showed a larger increase in cytotoxic activity than NK cells of healthy donors. **c**, cytotoxicity of IL-15 activated NK cells correlated with level of NKG2D expression on the CD56dim subset (Pearson correlation coefficient). **d**, primary osteosarcoma cell cultures were tested for sensitivity to lysis by autologous (○) and allogeneic (●) NK cells. NK cells were unstimulated (solid lines) or 3 days IL-15 activated (dashed lines). Autologous IL-15 activated NK cells were available for all patients except L2792. One way analysis of variance (ANOVA) *P*-value <0.0001 for a, b, and c. Bonferroni's multiple comparison post-test; *P*-value <0.05 noted as *; <0.01 = **; <0.001 = ***

better than healthy donor NK cells (ANOVA, Bonferroni's post-test *P*-value <0.0001). Percentage specific lysis of HOS correlated with the level of NKG2D expression on CD56 dim NK cells (Fig. 5.5c; Pearson correlation efficient r^2 0.45, *P*-value<0.0001) and similar results were obtained for the correlation with Granzyme B expression (not shown). To test whether the functional integrity of NK cells from osteosarcoma patients was also preserved towards autologous tumor cells, we took advantage of the fact that we were able to derive short-term cultured cells from fresh biopsies. Autologous, patient derived NK cells lysed short-term cultured tumor cells to a similar degree as allogeneic NK cells from healthy controls (Fig. 5.5d). In all cases, culture of both autologous and allogeneic NK cells in IL-15 resulted in greatly enhanced tumor cell killing.

DISCUSSION

There is increasing interest in the potential for NK cells to be used in the treatment of pediatric solid tumors [41]. Previous studies have shown that osteosarcoma cell lines may be sensitive to cytokine activated NK cell mediated cytotoxicity [104;132;143;156-159;165;191]. However, little is known about the mechanisms involved or the extent to which short-term cultured or chemotherapy resistant osteosarcoma cells are susceptible to NK cell mediated lysis. In addition, there is some evidence for NK cell mediated anti-osteosarcoma activity *in vivo*. Post-operative osteomyelitis-associated inhibition of tumor growth was dependent on the activation of monocytes and NK cells in a murine osteosarcoma model [242]. In human osteosarcoma, treatment with interleukin (IL)-2 in a small cohort of patients resulted in NK cell activation which was correlated with better clinical outcome [151]. Together, these studies suggest that exploitation of NK cell activity may be a suitable therapeutic tool in the adjuvant treatment of osteosarcoma. In the present study, we demonstrate that osteosarcoma cells are highly susceptible to NK cell mediated cytolysis (Fig. 5.1). Osteosarcoma cells expressed activating NKG2D and DNAM-1 ligands *in vivo* as well as *in vitro* and lysis was dependent on the interaction between these ligands on osteosarcoma cells and their receptors on NK cells (Figs. 5.2 and 5.3). Despite expression of the potentially inhibitory KIR ligand HLA class I by osteosarcoma cells, all cell lines and short-term cultures were highly sensitive to lysis by IL-15 activated NK cells (Table 5.1 and Figs. 5.1 and 5.5d). Together, these data suggest that the balance between expression of activating and inhibitory ligands in osteosarcoma is shifted towards activation.

To investigate if NK cell based immunotherapy is also feasible for patients with chemotherapy resistant disease, we tested the susceptibility of *in vitro* selected chemotherapy resistant osteosarcoma cells to NK cell mediated lysis. Methotrexate (MTX), doxorubicin (DX) or cisplatin (CDDP) resistant variants of the cell lines SAOS-2 and U2-OS remained sensitive to lysis by IL-15 activated NK cells (Fig. 5.4a). NK cells kill their targets by the release of perforin and granzyme containing granules and by the ligation of death receptors such as CD95 (Fas) [44;234]. Expression of CD95 was lost in the CDDP and DX resistant SAOS-2 variants (Table 5.1 and Fig. 5.4), but dual blocking studies demonstrated only a minor role for Fas ligation in the lysis of osteosarcoma by cytokine activated NK cells. Expression of Fas is frequently lost in osteosarcoma pulmonary metastases, but our data shows that this will probably not hinder NK cell based immunotherapeutic approaches [86;87;130].

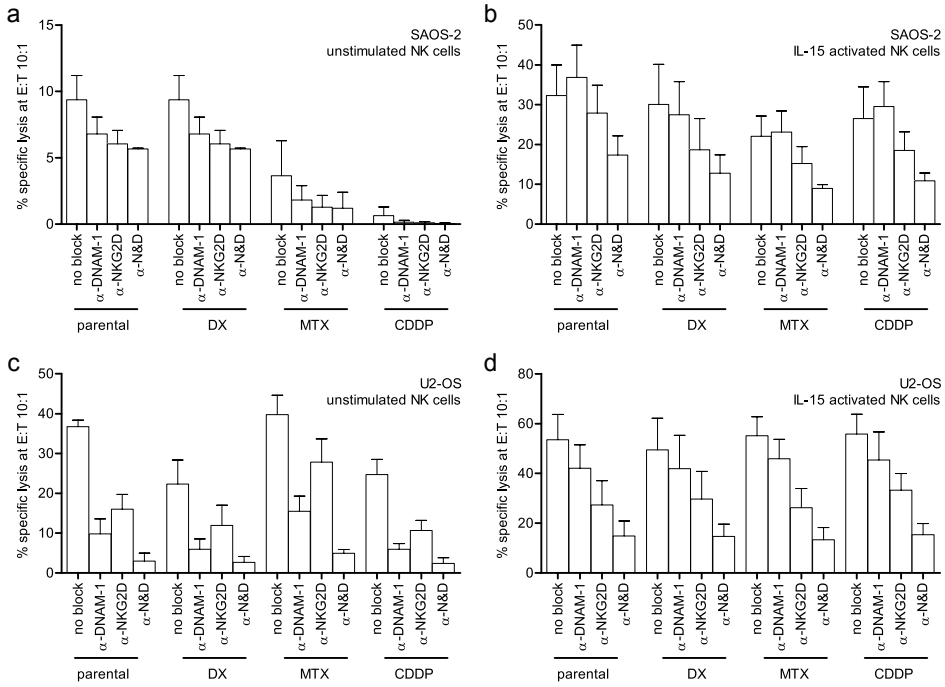
Studies on the feasibility of immunotherapeutic strategies in bone tumors are often hampered by the technical difficulty to isolate viable fresh tumor cells for functional testing. To circumvent this problem, we used short-term cultured cells. Susceptibility to NK cell mediated lysis was determined no later than at passage three. Four out of five cultures originated from patients with chemotherapy resistant disease (L2531, L2792, L2599 and L2808). Still, all were highly sensitive to lysis by cytokine activated autologous and allogeneic NK cells (Fig. 5.5d). Importantly, our experiments on *in vitro* selected chemotherapy resistant cells and on short-term cell cultures generated from patients with chemotherapy resistant disease *in vivo* show that IL-15 activated NK cells are capable of lysis of osteosarcoma cells resistant to chemotherapeutic agents commonly used in high-grade osteosarcoma treatment.

In many tumor types, including Ewing sarcoma, host immune cells have decreased functionality as compared to healthy donor cells [80;128;272]. In these cases, using allogeneic immune cells instead of autologous cells is an attractive option to increase efficacy. However, it also increases the risk of serious complications such as graft-versus-host-disease. Our data shows that NK cells of osteosarcoma patients are as potent as NK cells of healthy controls in lysing osteosarcoma cells. Remarkably, upon activation with IL-15, patient derived NK cells even showed a larger increase in expression of NKG2D and GranzymeB than healthy donor derived NK cells, which correlated with an increased lysis of the osteosarcoma cell line HOS (Fig. 5.5a and Suppl. Fig. 5.2c). This, and the lysis of autologous tumor cells by *ex vivo* IL-15 activated NK cells, indicates that immunotherapeutic strategies employing activated autologous NK cells could be as successful as allogeneic NK cells in the treatment of osteosarcoma. In preclinical validation studies we obtained evidence that IL15 and IL2 stimulated NK cells have similar cytolytic activity against various tumor cell lines [268]

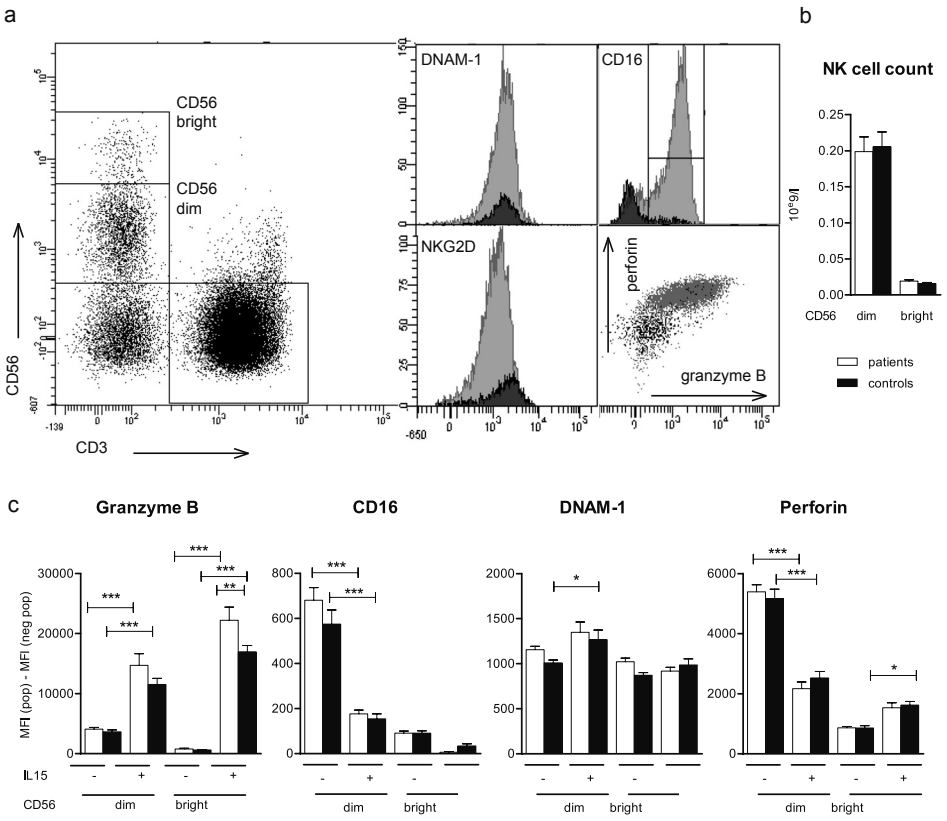
In conclusion, chemotherapy resistant and sensitive osteosarcoma cells were lysed at high levels by NK cells, particularly when NK cells were cytokine activated. Lysis of osteosarcoma cells was dependent on DNAM-1 and NKG2D, ligands of which were expressed by osteosarcoma cells both *in vivo* and *in vitro*. In contrast to what has been reported in patients with other tumor types, there was no intrinsic functional NK cell defect which could hamper anti-tumor activity. Our study shows a potential benefit of either activating NK cells *in vivo* by the administration of cytokines or adoptive transfer of *ex vivo* activated autologous or allogeneic NK cells in the treatment of high-grade osteosarcoma.

ACKNOWLEDGEMENT

This study is supported by a grant from the European Commission (EuroBoNeT, grant No 018814). EPB is funded by a grant from The Netherlands Organization for Health Research and Development (ZonMw grant 920-03-399). The authors wish to thank Monique van Ostaijen-ten Dam for help in the cytotoxicity assays, Maarten van Tol for fruitful discussions and provision of healthy donor PBMCs, Daniëlle de Jong for establishment and characterization of short-term tumor cell cultures and Nicolette Leyerzapf, Judith Kroep and Jakob Anninga for obtaining informed consent of osteosarcoma patients.



Suppl. Fig. 5.1 The relative contribution of NKG2D and DNAM-1 ligands to the lysis of SAOS-2 and its chemotherapy resistant variants was similar, as demonstrated by blocking DNAM-1, NKG2D or both receptors on unstimulated (**a**) and IL-15 activated (**b**) NK cells. Similar results were obtained in U2-OS (**c** and **d**). Error bars represent standard error of the mean lysis in at least three independent experiments using healthy donor NK cells. DX; doxorubicin resistant variant, MTX; methotrexate resistant variant, CDDP; cisplatinum resistant variant.



Suppl. Fig. 5.2 **a**, representative example of flow cytometry results of unstimulated peripheral NK cell compartment. The CD3 CD56 plot was gated on lymphocytes based on forward and sideward scatter. Monocytes were gated out by excluding CD14 stained cells (not shown). NK cells were defined as CD3-CD56+ cells and were divided in a CD56 bright and a CD56 dim subset. Expression of NKG2D, DNAM-1, CD16, perforin and granzyme B was determined for CD56 bright (black histogram and dots) and CD56 dim (grey histogram and dots) subsets. **b**, patients and controls had similar numbers of NK cells. **c**, following culture for 3 days in IL15, there was a larger increase in expression level of Granzyme B in NK cells of patients than of healthy controls. Patients and controls had similar levels of CD16, DNAM-1 and perforin on NK cells. In both patients and healthy controls, levels of CD16 decreased in CD56dim and bright NK cells and levels of perforin increased in CD56bright and decreased in CD56dim NK cells upon IL-15 treatment. ANOVA, *P*-value <0.0001. Bonferroni's multiple comparison post-test; *P*-value <0.05 noted as *; <0.01 = **; <0.001 = ***.

Suppl. Table 5.1 Composition of osteosarcoma tissue array

Number of samples (of nr of patients)	Type of sample
73 (73)	Pre-treatment samples of the primary tumor
45 (45)	Post-treatment samples of the primary tumor
20 (13)	Lung metastases
3 (3)	Other metastases (2 bone and one lymph node metastasis)
3 (3)	Local relapses
144 (88)	Total samples

Suppl. Table 5.2 Clinicopathological details of patients in this study

		Peripheral blood NK cell analysis	
	Tissue array	**Osteosarcoma patients**	**Healthy donors**
Total nr of patients	88	22	23
Age in years: median (range)	15.4 (4-44)	16 (9-56)	20 (8-55)
Sex	n (%)	n (%)	n (%)
Male	47 (53.4%)	10 (45.5%)	11 (47.8%)
Female	41 (46.6%)	12 (54.5%)	12 (52.2%)
Location of primary tumor			
Distal femur	40 (45.5%)	11 (50%)	
Proximal tibia/fibula	27 (30.7%)	4 (18.2%)	
Proximal humerus	10 (11.4%)	4 (18.2%)	
Other long bones	9 (10.2%)	3 (13.6%)	
Axial skeleton	1 (1.1%)	0	
Hand	1 (1.1%)	0	
Histological subtype			
Conventional osteosarcoma	71 (80.7%)	18 (81.8%)	
Osteoblastic	59	12	
Chondroblastic	9	3	
Fibroblastic	3	1	
Unusual	8	2	
Telangiectatic	6 (6.8%)	4 (18.2%)	
High-grade surface osteosarcoma	1 (1.1%)	0	
Small cell osteosarcoma	2 (2.3%)	0	
Date of diagnosis:	Jan 1981- Sep 2003	Feb 2007 - Apr 2008	
Treated according to protocols:	EORTC 80831, 80861 and 80931	Euramos-1	

Suppl. Table 5.3 Clinicopathological details of primary cultures

Primary culture	**Origin of primary culture**	**Sex**	**Age (y)**	**Histological subtype of osteosarcoma**	**Location of primary tumor**
L2531	Local relapse	Male	17	Osteoblastic	Distal femur
L2808	Lung metastasis	Male	18	Osteoblastic	Distal femur
L2792	Local relapse	Female	31	Fibroblastic	Proximal humerus
L2599	Diagnostic biopsy	Male	13	Osteoblastic	Distal femur
L2635	Diagnostic biopsy	Female	14	Osteoblastic-sclerosing type	Distal femur

Suppl. Table 5.4 Antibodies used for immunohistochemistry (IHC) and flow cytometry (FC)

Antibody	Catalog/ clone nr	Company	Application
b2-microglobulin	A0072	DAKO (Glostrup, Denmark)	IHC, antigen retrieval (AR): Citrate, 0.01 M, pH 6
HLA-A	HCA-2	Kindly provided by J.Neefjes (NKI, Amsterdam, the Netherlands)	IHC, AR: Citrate, 0.01 M, pH 6
HLA B/C	HC10	Kindly provided by J.Neefjes	IHC, AR: Citrate, 0.01 M, pH 6
MICA	AF1300	R&D systems (Minneapolis, MN)	IHC, AR: Citrate, 0.01 M, pH 6
CD155	HPA012568	Sigma Aldrich (Zwijdrecht, the Netherlands)	IHC, AR: EDTA, 1 mM, pH 8
ULBP-1	HPA007547	Sigma Aldrich	IHC, AR: Citrate, 0.01 M, pH 6
CD112	HPA012759	Sigma Aldrich	IHC, AR: EDTA, 1 mM, pH 8
MICA	MAB1300	R&D systems	FC, cell lines
MICB	FAB1599A	R&D systems	FC, cell lines
ULBP-1	IC1380P	R&D systems	FC, cell lines
ULBP-2	FAB1298A	R&D systems	FC, cell lines
ULBP-3	MAB1517	R&D systems	FC, cell lines
CD48-PE	IM1837U	Beckman Coulter Immunotech (Marseille, France)	FC, cell lines
CD155	IM2755	Beckman Coulter Immunotech	FC, cell lines
CD112-PE	IM3452	Beckman Coulter Immunotech	FC, cell lines
HLA-A/B/C-FITC	555552	BD Pharmingen (San Diego, CA)	FC, cell lines
CD54-PE	555511	BD Pharmingen	FC, cell lines
CD58-PE	555921	BD Pharmingen	FC, cell lines
CD95-PE	340480	BD Pharmingen	FC, cell lines
goat anti-mouse APC	550826	BD Pharmingen	FC, secondary antibody
mIgG1-FITC	639	Beckman Coulter Immunotech	FC, isotype control
mIgG2b	X0944	DAKO	FC, isotype control
mIgG2a	MAB0031	R&D	FC, isotype control
mIgG1-PE	349053	BD Pharmingen	FC, isotype control
mIgG1	MAB002	R&D	FC, isotype control
mIgG2a-PE	349053	BD Pharmingen	FC, isotype control
CD3-PerCPCy5.5	332771	BD Pharmingen	FC, purity of isolated NKs
CD20-FITC	345792	BD Pharmingen	FC, purity of isolated NKs
CD14-APC	340436	BD Pharmingen	FC, purity of isolated NKs
CD56-PE	R7251	DAKO	FC, purity of isolated NKs
CD3-Pacific Blue	558117	BD Pharmingen	FC, PBMCs
CD14-PerCPCy5.5	550787	BD Pharmingen	FC, PBMCs
DNAM-PE	559789	BD Pharmingen	FC, PBMCs
GranzymeB-Alexa700	557971	BD Pharmingen	FC, PBMCs
NKG2D-APC	558071	BD Pharmingen	FC, PBMCs
CD56-PECy7	A21692	Beckman Coulter Immunotech	FC, PBMCs
CD16-FITC	IM0814	Beckman Coulter Immunotech	FC, PBMCs
Perforin-FITC	358-040	Hölzel Diagnostika, Cologne, Germany	FC, PBMCs

6.

Intact interferon signaling in peripheral blood leukocytes of high-grade osteosarcoma patients

Emilie P. Buddingh, S. Eriaty N. Ruslan, Dagmar Berghuis, Hans Gelderblom, Jakob K. Anninga, Pancras C.W. Hogendoorn, R. Maarten Egeler, Marco W. Schilham, Arjan C. Lankester

Cancer Immunol Immunother. 2012 Jun;61(6):941-7.

ABSTRACT

High-grade osteosarcoma has a poor prognosis with an overall survival rate of about 60 percent. The recently closed European and American Osteosarcoma Study Group (EURAMOS)-1 trial investigates the efficacy of adjuvant chemotherapy with or without interferon-α. It is however unknown whether the interferon-signaling pathways in immune cells of osteosarcoma patients are functional. We studied the molecular and functional effects of interferon treatment on peripheral blood lymphocytes and monocytes of osteosarcoma patients, both *in vivo* and *ex vivo*. In contrast to other tumor types, in osteosarcoma, interferon signaling as determined by the phosphorylation of signal transducer and activator of transcription (STAT)1 at residue 701 was intact in immune cell subsets of 33 osteosarcoma patients as compared to 19 healthy controls. Also, cytolytic activity of interferon-α stimulated natural killer cells against allogeneic (n = 7 patients) and autologous target cells (n = 3 patients) was not impaired. Longitudinal monitoring of three osteosarcoma patients on interferon-α monotherapy revealed a relative increase in the CD16-positive subpopulation of monocytes during treatment. Since interferon signaling is intact in immune cells of osteosarcoma patients, there is a potential for indirect immunological effects of interferon-α treatment in osteosarcoma.

INTRODUCTION

High-grade osteosarcoma is a primary malignant bone tumor with an overall survival rate of about sixty percent [8]. Intensification of chemotherapeutic treatment has not led to improved outcome, and other therapeutic modalities are currently under investigation [141]. The recently closed for accrual European and American Osteosarcoma Study Group (EURAMOS)-1 trial investigates whether maintenance treatment with interferon(IFN)-α-2b after standard chemotherapy for osteosarcoma patients who have a good response to preoperative chemotherapy (i.e., >90% necrotic tumor tissue) is effective [160;278]. IFN-α has proven anti-tumor effect in several tumor types, such as hairy cell leukemia and renal cell cancer, and has been used in osteosarcoma patients as adjuvant treatment in Scandinavia since the 1970s [163;168;249].

The anti-proliferative effect of IFN-α on osteosarcoma cells has been shown *in vitro* and in a xenograft model in nude mice [31;154]. Also, expression of IFN receptors on human osteosarcoma cells is associated with a better prognosis [133]. However, IFN-α can also exert indirect anti-tumor activity; for example, through immunostimulatory effects. The type I IFNs (IFN-α and -β) binds to the IFN type I receptor consisting of the two subunits IFNAR1 and IFNAR2, which is expressed by immune cells such as natural killer (NK) cells and monocytes [100;207]. Indirect anti-tumor effects of type I IFNs were essential for the clearance of immunogenic sarcomas in IFNAR1 deficient mice, since it was dependent on the expression of IFNAR1 on hematopoietic host cells and not on tumor cells [61]. Whether similar indirect anti-tumor effects also occur in the treatment for human osteosarcoma with type I IFNs is unknown. The binding of type I and type II (IFN-γ) IFNs to their respective receptors results in the activation of Janus Kinase (JAK), subsequent phosphorylation of signal transducer and activator of transcription (STAT) and finally transcription of target genes. Phosphorylation of STAT1 at tyrosine residue 701 occurs rapidly following receptor–ligand interaction and is critical for both type I and type II IFN signaling [54]. IFN signaling as determined by STAT1 phosphorylation was impaired in lymphocytes of patients suffering from breast cancer, melanoma, and gastrointestinal cancer [27;51;52;62]. Impaired IFN signaling may be rescued at the level of JAK-1 induced STAT1 phosphorylation; for example, by interleukin (IL)-12 pre-treatment, as was shown in a murine melanoma model [139].

Here, we addressed the molecular and functional effects of IFN treatment on immune cell subsets of osteosarcoma patients, both *in vivo* and *ex vivo*. To interpret the future results of the IFN-α treatment arm of the EURAMOS-1 trial, it is essential to know whether IFN-signaling pathways in immune cells of osteosarcoma patients are intact.

PATIENTS AND METHODS

Patients

Peripheral blood mononuclear cells (PBMCs) of 33 newly diagnosed osteosarcoma patients and 19 healthy controls were available for flow cytometric evaluation of IFN-induced phosphorylation of STAT1 (Table 6.1). PBMCs of 7 patients and 7 controls were available for cytolytic experiments. From three osteosarcoma patients treated with IFN-α monotherapy following the completion of adjuvant chemotherapy, PBMCs were collected at diagnosis (prior to the start of chemotherapy),

Table 6.1 Clinicopathological details of osteosarcoma patients and healthy controls included.

	osteosarcoma	healthy controls
n	33	19
Male	18 (54,5 %)	12 (63.2 %)
age median (range)	16 (6-56)	17 (8-45)
location primary tumor		
distal femur	19 (57.6 %)	
prox tibia	6 (18.2 %)	
prox humerus	5 (15.2 %)	
other long bone	3 (9.1 %)	
histological subtype		
conventional	29 (87.9 %)	
osteoblastic	24	
chondroblastic	3	
unusual	2	
telangiectatic	4 (12.1 %)	
high grade surface	1 (3.0 %)	

prior to the start of IFN-α monotherapy and at one or two time points during the first few weeks of treatment with IFN-α (subcutaneous PegIntron, 0.5 µg/kg/week for 4 weeks, then dose escalation to 1.0 µg/kg/week). PBMCs were obtained after written informed consent, approved by the Institutional Review Board. All samples were handled in a coded fashion.

Culture of cell lines and PBMCs

The cell line K562 (obtained from ATCC) and PBMCs were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (FCS, Invitrogen) and 1% penicillin/streptomycin (P/S, Invitrogen). PBMCs were isolated by Ficoll density gradient centrifugation and stored in liquid nitrogen. After thawing, cells were allowed to recover overnight, except when used for flow cytometric evaluation of dendritic cell (DC) activation, in which case, cells were analyzed immediately. For cytolytic assays, cells were cultured overnight with or without 100 IU/mL IFN-α (Roche, Basel, Switzerland). Prior dose-finding pilot experiments determined this dose to result in good cell viability and reproducible NK cell activation (data not shown). The primary cell culture L2635 was established from a pre-treatment biopsy of osteosarcoma patient 398 as described previously and maintained in RPMI 1640 with 20% FCS and 1% P/S [34].

Flow cytometric evaluation of IFN-induced phosphorylation of STAT1

Cells were stained with surface staining antibodies in staining buffer (PBS with 0.05% bovine serum albumin without sodium azide) for 30 min at 37°C. Surface staining antibodies used for natural killer (NK) and T-cell subsets were fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (349201, BD, San Diego, CA) and allophycocyanin (APC)-conjugated anti-CD56 (2474,

IOtest/Immunotech, Marseille, France). Antibodies used for monocyte and B cell subsets were FITC-conjugated anti-CD20 and APC-conjugated anti-CD14 (345792 and 340436, BD). Cells were stimulated with or without 1,000 IU/mL IFN-α or IFN-γ for 15 min at 37°C and fixed in 4% paraformaldehyde, as previously described by Critchley-Thorne et al. [15]. Cells were permeabilized using ice-cold Perm Buffer III (BD Phosflow) for 30 min and stained for 60 min on ice with phycoerythrin (PE)-conjugated anti-STAT1 (directed against the N-terminus of STAT1 to determine total STAT1 levels, 558537, BD) and peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated anti-pY701-STAT1 (directed against phosphorylated tyrosine residue 701 of STAT1 to determine the levels of phosphorylated STAT1, 560113, BD). Isotype control antibodies were used to correct for the background levels of fluorescence. All flow cytometric analyses were done on a FACScalibur with Cellquest software (both BD).

Flow cytometric evaluation of PBMC subsets

PBMC subsets were determined as follows: T cells were CD3-PerCP-Cy5.5-positive (332771, BD), NK cells were CD3-PerCP-Cy5.5-negative, and CD56-PE-positive (R7251, Dako, Glostrup, Denmark), B-cells were CD20-FITC-positive (345792, BD), and monocytes were CD14-APC-positive (340436, BD). Myeloid dendritic cells (mDCs) were CD3-, CD19-, and CD14-negative (all PerCP-Cy5.5-conjugated, 332771, 332780, and 550787, BD) and BDCA-1- and BDCA-2-positive (PE-conjugated, Miltenyi Biotec, Bergisch Gladbach, Germany). Activation status of mDCs was determined by evaluating mean fluorescence intensities of HLA-DR-APC (347403, BD) and CD86-FITC (555657, BD). Plasmacytoid dendritic cells (pDCs) were CD3-, CD19-, and CD14-negative and BDCA-2-APC-positive (Miltenyi). Activation status of pDCs was determined with anti-CD86-FITC and anti-HLA-DR-PE (555657 and 347367, BD). Monocytes were evaluated by CD14-PerCP-Cy5.5, CD16-PE (347617, BD), HLA-DR-APC, and CD86-FITC.

Cytolytic assays

Four hour chromium release cytolytic assays were performed as described earlier [34]. Briefly, target cells were incubated with 3.7 MBq sodium-51-chromate (PerkinElmer, Wellesley, MA) for 1 h. Effector cells (with or without overnight IFN-α stimulation) were incubated for 4 h with 2,500 target cells at eight effector:target (E:T) ratios in triplicate. Using these conditions, cytolysis observed is caused by NK cells, since antigen-specific T cells (which are present at low frequencies if at all) are insufficiently expanded. Therefore, the E:T ratios were corrected for the percentage of NK cells of PBMCs as determined by flow cytometry. Maximum and spontaneous release was determined by incubating targets in 2N HCl or medium, respectively. Supernatants were harvested and counted in a gamma counter (Wallac, PerkinElmer). Specific lysis was determined as: (experimental release-spontaneous release)/(maximum release-spontaneous release) × 100%.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (LaJolla, CA). Two-sided *P* values lower than 0.05 were determined to be significant.

RESULTS AND DISCUSSION

Peripheral blood monocytes and lymphocytes of osteosarcoma patients have intact interferon-signaling *ex vivo*

Total STAT-1 levels were similar in peripheral blood subsets of osteosarcoma patients and healthy controls. To determine whether the IFN-signaling pathway was intact, we assessed STAT1 phosphorylation in IFN-stimulated immune cells of controls and patients. Basal levels of phospho-STAT1 were slightly lower in NK cells of osteosarcoma patients at diagnosis (n = 33) than in NK cells of healthy controls (n = 19; Fig. 6.1a), similar to what has been described for melanoma [140]. However, in contrast to what has been reported for melanoma, breast cancer, and gastrointestinal cancer, IFN signaling was intact in immune cells of osteosarcoma patients, as determined by a 15 min *ex vivo* stimulus with 1,000 IU/mL IFN-α or IFN-γ (results for IFN-α shown in Fig. 6.1, results for IFN-γ not shown). Responses were similar in almost all PBMC subsets for patients and controls, except for a hyperphosphorylation of STAT1 in B- and NK cells of osteosarcoma patients in response to IFN-α (Fig. 6.1b). The functional response of NK cells of osteosarcoma patients and healthy controls to IFN-α was similar, as both responded to overnight IFN-α culture with increased cytolytic capacity toward the NK cell target K562 (Fig. 6.1c). There was possibly some, but not statistically significant trend for correlation between phosphorylated STAT1 in NK cells and percentage of specific lysis of K562 (Fig. 6.1d, *P* value 0.06, Spearman r 0.52).

We previously showed higher cytolytic capacity of IL-15-activated NK cells of osteosarcoma patients than of healthy donors [34]. The binding of IL-15 and IFN-α to their respective receptors has the association of JAK1 with the receptors in common. Together with Tyk2, this results in phosphorylation of STAT1 in case of IFN-α-signaling and together with JAK3 in phosphorylation of STAT3 and STAT5 in case of IL-15 signaling. The hyperphosphorylation of STAT1 in response to IFN-α and the earlier reported increased cytolytic capacity in response to IL-15 in NK cells of osteosarcoma patients as compared to healthy donors could be due to increased functionality of the common upstream signaling molecule JAK1. Pro-inflammatory cytokines such as IL-12 potentiate JAK1 induced STAT phosphorylation [139]. However, levels of IL-12(p70) were similar in plasma of patients and controls, and IL-12(p70) levels did not correlate with IFN-α induced STAT1 phosphorylation (data not shown). Whether increased STAT1 phosphorylation in NK cells of osteosarcoma patients is related to differential expression or activity of JAK1 remains to be investigated.

Phenotypic and functional analysis of PBMCs of osteosarcoma patients on IFN-α monotherapy

In three osteosarcoma patients who received IFN-α monotherapy following chemotherapeutic treatment according to the EURAMOS-1 protocol, detailed flow cytometric phenotypic analysis of PBMCs was performed during the first few weeks of IFN-α monotherapy. In all three patients, B cell numbers were very low (on average 3% of lymphocytes) at the end of chemotherapeutic treatment and during IFN treatment returned to levels similar to those at diagnosis, before start of chemotherapy (on average about 30% of lymphocytes or 500 cells/μL). No consistent changes were seen in percentage or absolute number of mDCs, pDCs, NK-, or T cells. The

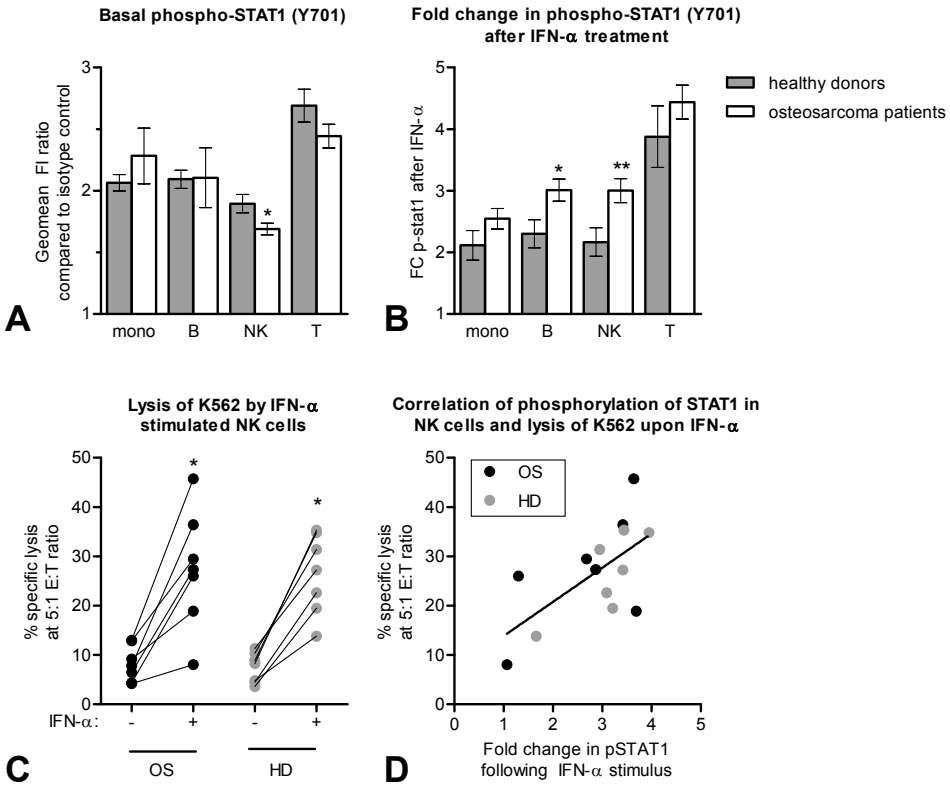


Fig. 6.1 PBMCs of osteosarcoma patients are normally responsive to IFN-α. **a**, flow cytometric evaluation of basal levels of phosphorylated STAT1 in immune cell subsets shows slightly lower levels of phosphorylated STAT1 in NK cells of osteosarcoma patients at diagnosis (n = 33) as compared to healthy donors (n = 19). **b**, following an *in vitro* stimulus with 1,000 IU/mL IFN-α, hyperphosphorylation of B and NK cells of osteosarcoma patients was observed. For both a and b, error bars denote standard error of the mean; student's t-test *P* value <0.05 noted as *; <0.01 = **. **c**, lysis of the allogeneic NK cell target K562 by PBMCs of healthy donors (HD) and osteosarcoma patients (OS) increased significantly following overnight culture in 100 IU/mL IFN-α (IFN-α +). Effector to target (E:T) ratio was corrected for percentage of NK cells. Lysis was similar by healthy donor and osteosarcoma patient derived cells. Wilcoxon signed ranked test; *P* value <0.05 noted as *. **d**, following overnight culture of PBMCs in IFN-α, percentage-specific lysis correlated with fold change in pSTAT1 in NK cells (*P* value 0.06, Spearman r 0.52).

activation status of monocytes, mDCs, or pDCs did not change during the first weeks of treatment, as measured by the expression of CD86 and HLA class II.

In all three patients, a reduction in percentage of monocytes was seen during the first 2–8 weeks of IFN treatment. However, the relative contribution of CD16+ monocyte subpopulation increased (Figs. 6.2a, b). CD16+ monocytes are considered "pro-inflammatory" because of the high expression of tumor necrosis factor (TNF)-α and low expression of IL-10 [287]. These pro-inflammatory monocytes may have anti-tumor effects, akin to what has been described for "pro-inflammatory" M1 type macrophages. We have recently demonstrated the prognostic significance of infiltrating macrophages in osteosarcoma [33]. Further studies are needed

to determine whether IFN-induced phenotypic changes in peripheral blood monocytes are associated with changes in intratumoral macrophage number or phenotype, and whether this is associated with altered expression of specific cytokines. However, this will require IFNs to be administered earlier, before resection of the primary tumor or relapses.

During *in vivo* treatment with IFN-α, total STAT1 levels in monocytes, NK cells, and T cells increased, but the basal levels of phosphorylated STAT1 did not change in any of the leukocyte subsets (data not shown). NK- and T cells, which had been exposed to IFN-α *in vivo*, showed less induction of phosphorylation of STAT1 in response to *ex vivo* IFN-α stimulation (NK cells shown in Fig. 6.2c). Nevertheless, induction of cytolytic capacity against the NK cell target K562 and the osteosarcoma culture L2635 following overnight IFN-α stimulation of PBMCs was unimpaired, both against autologous and allogeneic targets (example shown for PBMCs of patient 398 and its autologous target cells L2635 in Fig. 6.2d), indicating that *in vivo* treatment with IFN-α has not rendered these cells functionally unresponsive to an additional *ex vivo* IFN stimulus.

The lack of induction of phosphorylated STAT1 during *in vivo* IFN-α treatment was surprising. There are several possible explanations. Phosphorylation of STAT1 is an event that occurs rapidly following IFN stimulus. Perhaps, STAT1 phosphorylation *in vivo* can be observed when PBMCs are analyzed within a few hours of first administration of IFN. Further studies are needed if this is indeed the case and if so, if this supposed the early activation of the JAK/STAT pathway in immune cells results in sustained activation of target genes and functional pathways. An alternative explanation might be that the level of IFN-induced activation of immune cells *in vivo* is dose-dependent and limited by toxic and adverse effects *in vivo* [7]. An additional *in vitro* stimulus was able to induce STAT1 phosphorylation and resulted in increased cytolytic NK cell activity, suggesting that *in vivo* IFN-α-induced immune cell activation might have been incomplete. On the other hand, it is still possible that the prolonged, relatively low level of IFN-α exposure *in vivo* on PBMCs does result in sustained anti-tumor immune effects; for example, through the observed changes in monocytes.

CONCLUDING REMARKS

Treatment for osteosarcoma patients with the anti-proliferative and immunomodulatory agent IFN-α could have direct anti-tumor and/or indirect immune-mediated effects. Here, we show IFN signaling to be intact in PBMCs of osteosarcoma patients, as determined by the phosphorylation of STAT1 and increased NK cell-mediated cytolysis in response to *ex vivo* IFN. Some of the experiments were performed on small sample numbers, but since the results were consistent throughout, we believe the results to be valid in determining responsiveness of immune cells of osteosarcoma patients to IFN-α. Although the efficacy of IFN-α treatment in osteosarcoma will be assessed in the EURAMOS-1 trial, our data indicate that in contrast to some other tumor types, immune cells of osteosarcoma patients have retained IFN-responsiveness. Further clinical studies are needed to establish the extent and efficacy of indirect immunotherapeutic effects of IFN-α treatment in osteosarcoma patients.

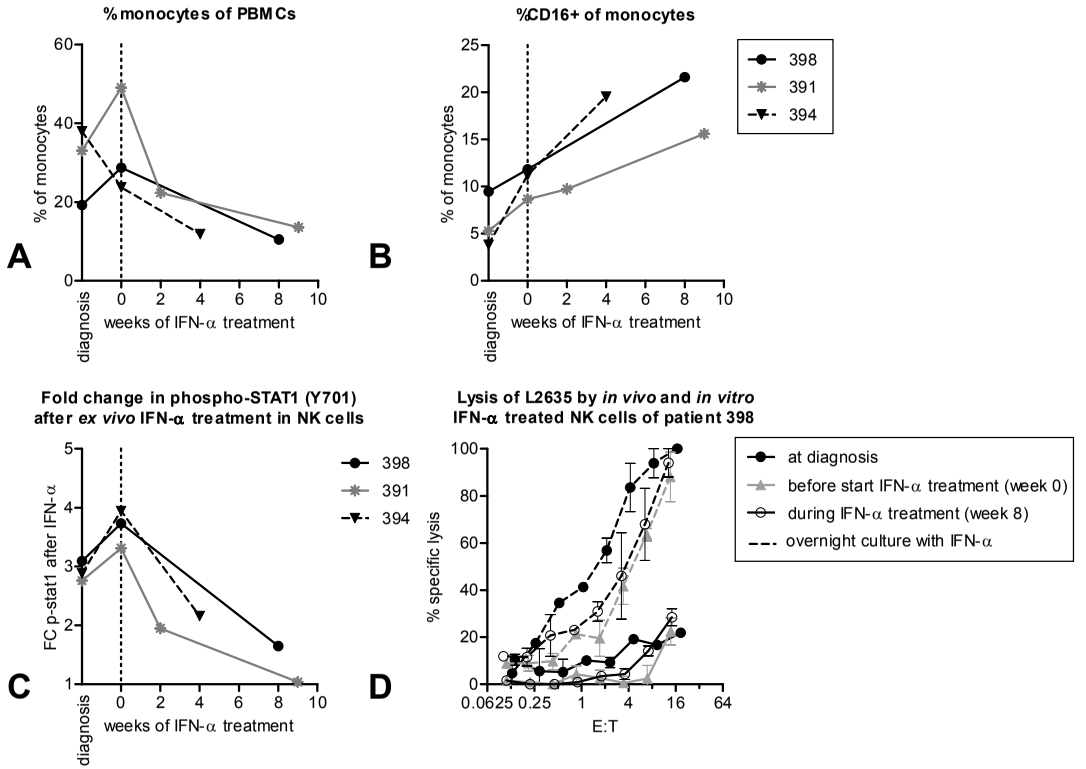


Fig. 6.2 Effect of in vivo IFN-α administration on monocytes and NK cells. **a**, following *in vivo* treatment with IFN-α of three osteosarcoma patients, the percentage of monocytes of PBMCs decreased. **b**, the relative percentage of CD16-positive monocytes increased. **c**, NK cells which had been exposed to IFN-α *in vivo* showed less phosphorylation of STAT1 in response to *ex vivo* IFN-α stimulation. **d**, cytolysis of the autologous osteosarcoma target cell line L2635 by PBMCs of patient 398 did not change significantly during *in vivo* treatment with IFN-α (solid lines). Following overnight culture in 100 IU/mL IFN-α, lysis by PBMCs collected at all time points increased (dashed lines). Effector to target (E:T) ratio was corrected for the percentage of NK cells. Error bars denote standard error of the mean of experiment performed in triplicate.

ACKNOWLEDGMENTS

Financial support: This work was financially supported by a grant from the foundation "Quality of Life Gala 2007" and the Netherlands Organization for Health Research and Development [ZonMw, grant 92003-399 to EPB].

7.

Discussion and future prospects

Part of this chapter has been adapted from:

Immunotherapy: Is it different for sarcomas?
Anne-Marie Cleton-Jansen, Emilie P. Buddingh, Arjan C. Lankester

Oncoimmunology. 2012 Mar 1;1(2):255-257

High-grade osteosarcoma is a malignant bone tumor with the highest incidence in young patients. The prognosis for patients with metastasized disease remains dismal, despite aggressive surgery and intensive chemotherapeutic regimens. In **chapters 2** and **3** of this thesis, studies aimed at a better understanding of the etiology and prognostic factors of high-grade osteosarcoma are described. In **chapters 4**, **5** and **6** interactions between high-grade osteosarcoma cells and innate immune cells are studied. Together, these studies can guide the design of clinical studies implementing novel immunotherapeutic regimens. Adding immunotherapy to standard treatment regimens will hopefully result in better survival for osteosarcoma patients.

ETIOLOGY AND PROGNOSTIC FACTORS OF HIGH-GRADE OSTEOSARCOMA

The cell of origin of high-grade osteosarcoma remains elusive, but the strong temporal and spatial relationship with proliferating and differentiating mesenchymal stromal cells (MSCs), suggests that MSCs or early osteoblast precursor cells are likely suspects. Additionally, long term *in vitro* expansion of murine mesenchymal stem cells results in spontaneous oncogenic transformation of the cultured cells which form osteosarcoma-like tumors *in vivo* [113;116;179;183;259]. These transformed cells have a near-tetraploid aneuploid karyotype and show loss of the CDKN2 locus, similar to human spontaneous osteosarcoma [183]. MSCs derived from cynomolgus macaques also transform upon long term *in vitro* culture [107]. Previously, some groups have reported spontaneous transformation of human MSCs, only to later retract these publications because of contamination of the cultures with other cell lines [56;218;221-223;260]. In childhood leukemias, preleukemic alterations have been found in hematopoietic stem cells years before the leukemia developed [164]. The acquisition of additional mutations and subsequent clonal evolution results in the outgrowth of a true leukemic clone [110]. Similarly, in solid tumors, the 'multiple hit' hypothesis postulates that several chronological mutational and cell cycle deregulatory events are necessary for cancer to develop [66]. Alternatively, a single catastrophic event, termed chromothripsis, can result in highly complex chromosomal rearrangements and oncogenic transformation [102;247]. This probably has to occur in a susceptible background, either acquired in specific individual cells or as a somatic genetic predisposition. In **chapter 2**, we hypothesized that MSCs of osteosarcoma patients might harbor one or a few of such 'pre-cancerous' alterations. The added cellular stress of prolonged *in vitro* culture could then result in additional (pre-) oncogenic hits and a higher propensity to spontaneous transformation in osteosarcoma patient derived MSCs than in MSCs harvested from healthy donors. Since MSCs originating from the tumor site but predating tumor formation were obviously not available, we chose to study MSCs harvested from the iliac crest at diagnosis, prior to chemotherapeutic treatment.

On a transcriptional level, downregulation of hematopoietic cell specific Lyn substrate 1 (*HCLS1*) was noted in osteosarcoma patient derived MSCs as compared to healthy donor derived MSCs, the product of which is involved in B-cell receptor signaling and myelopoiesis [75;233]. If downregulation of *HCLS1* in osteosarcoma patient derived MSCs has a functional role in osteosarcomagenesis is as yet unknown. Despite almost two years in culture, none of the samples underwent spontaneous transformation. An increase in binucleation was noted upon increasing passage in both osteosarcoma patient and healthy donor derived MSCs. Perhaps the binucleation is a result of telomere shortening, anaphase bridges and failed cytokinesis [77;123;201]. In any case, functional cell cycle checkpoints were apparently intact in both patients and healthy donors, as no transformation occurred and cytogenetic analysis on metaphases did not reveal any karyotypic abnormalities.

As was discussed in **chapter 2**, there are several possible explanations for the observed similarities between MSCs derived from osteosarcoma patients and healthy donors. It is possible that the hypothesis that osteosarcoma derives from MSCs is incorrect. Alternatively, MSCs are

the precursor cells, but (pre-)oncogenic alterations are not present in MSCs harvested from the iliac crest. This does not preclude the presence of preoncongenic alterations in MSCs at other sites. For example, there could be somatic mosaicism, similar to what has been shown for the enchondromatosis syndromes Ollier disease and Maffucci syndrome [202]. Another possibility is that prolonged *in vitro* culture is insufficient for oncogenic transformation to occur. According to the 'multiple hit hypothesis', several oncogenic alterations are necessary for transformation to occur. Perhaps growth factor signaling (endocrine and paracrine stimuli in the microenvironment where osteosarcoma arises, *i.e.* near the growth plate) or additional oncogenic 'hits' such as loss of cell cycle control are a prerequisite for oncogenic transformation of susceptible MSCs. For example, prolonged culture of MSCs on a background of downregulated CDKN2/p16 may yield transformed cells. P53 is essential for preventing cell cycle progression in case of failed cytokinesis. Loss of p53 might result in cell cycle progression in case of failed cytokinesis with tetraploidy and aneuploidy as a result [77]. A better understanding of what drives oncogenic transformation in human MSCs will help elucidate the mechanisms important in osteosarcomagenesis, which in turn has the potential to identify targets for therapy.

Pulmonary metastatic disease is the main cause of death for osteosarcoma patients [24]. About one in five osteosarcoma patients present with clinically evident pulmonary metastatic disease at diagnosis and two in five develop pulmonary metastatic disease during treatment or follow up of their disease. Risk factors associated with the development of pulmonary metastases include a poor histological response to pre-operative chemotherapy and the presence of a primary tumor not amenable to local resection. Despite aggressive multimodal therapy, about four in five patients with pulmonary metastatic disease succumb to their disease. It is these patients that would benefit most from novel adjuvant (immuno-)therapies.

In **chapter 3**, prognostic factors related to the survival of patients with pulmonary metastasized high-grade osteosarcoma were studied. Higher metastatic tumor burden (*i.e.* larger number of pulmonary nodules), presence of vital metastases upon resection and male sex were associated with an increased risk of death. In **chapter 4**, genome-wide expression studies were performed to identify genes associated with a risk for pulmonary metastatic disease. High expression of macrophage-associated genes was associated with a lower risk of metastatic disease (discussed in paragraph 7.2.1). Together, the results of **chapters 3** and **4** show that osteosarcoma patients with morphologically vital metastases and a high metastatic tumor burden may benefit from immunotherapeutic strategies exploiting migration and activation of monocytes/macrophages towards the tumor site.

INTERACTIONS BETWEEN HIGH-GRADE OSTEOSARCOMA CELLS AND INNATE IMMUNE CELLS

Tumor-associated macrophages in high-grade osteosarcoma

The Janus-faced roles of macrophages in cancer imply both tumor-suppressive and -stimulating actions of these innate immune cells. Whereas the balance is toward tumor promotion in most

epithelial cancers, in **chapter 4** we show that osteosarcoma metastasis seems to be inhibited by the presence of macrophages in the tumor microenvironment.

It is not a coincidence that the 'Father of Immunotherapy,' William B. Coley, was a bone sarcoma surgeon. The first successful example of immunotherapy was in 1891 when Coley's toxins, a mixture of toxins of streptococcal bacteria was injected into an unresectable sarcoma. The resulting immunological reaction led to tumor regression, similar to what has been observed in osteosarcoma patients suffering from post-operative infection following resection of their primary tumor [49]. The few known permanent responses to Coley's toxins in carcinoma were in those cases of mesodermal origin [246]. Are sarcomas and other tumors of mesodermal origin more immunogenic than carcinomas? Or do immune cells have an effect that is different between sarcomas and carcinomas?

The tumor promoting effect of macrophages in carcinomas is well established. Epithelial tumors with high numbers of infiltrating immune cells have a poor prognosis as compared with cases with few infiltrating cells. This is attributed to a number of properties of the immune cells, especially macrophages, which have been shown to be involved with tumor initiation, invasion, migration, intravasation and angiogenesis [210]. Especially the stimulating effect on tumor invasion and migration of in origin non-motile epithelial cells that are the progenitors of carcinomas can be well comprehended. However, it is different for mesenchymal cells, which are much less dependent on contact with adjacent cells and thus more motile. These cells probably do not need the guidance that immune cells seem to give to carcinoma cells in the circulation. Instead, mesenchymal tumor cells might be inhibited in their motility by macrophages, which then act as impediment, instead of promoter for invasion. This is of course speculative, but it has been reported that macrophage inhibitory factor, MIF, which is produced by macrophages, inhibits migration of mesenchymal stem cells [69].

In **chapter 4**, an expression profiling study in a relatively large series of high-grade osteosarcomas was performed, with results corroborating a metastasis inhibiting role for macrophages. The 'expression profile' associated with non-metastatic behavior of osteosarcoma surprisingly consisted of a large number of genes associated with macrophage function, such as antigen processing and presentation or pattern recognition, as well as specific monocyte and macrophage markers such as *CD14* and *MSR1*. Also a large number of genes with other immunological functions, such as cytokine production and phagocytosis were found to be upregulated. Expression of the macrophage-associated genes was confined to primary tumor tissue and not detected in a panel of 19 osteosarcoma cell line RNA samples, indicating that infiltrating immune cells were responsible for this expression profile. Furthermore the results were confirmed at the protein level by immuno histochemical staining on a larger patient cohort.

A role for macrophages to prevent or reduce metastases of osteosarcoma is corroborated by one of the few efficacious new therapeutic agents that were tested since the successful introduction of conventional chemotherapy for osteosarcoma, i.e., liposomal muramyl tri-peptide (MTP), also known as Mepact® or mifamurtide. This proprietary drug elicits activation of macrophages. Although the clinical trial that included adjuvant treatment with mifamurtide was initially denounced because of a presumed interaction with one of the chemotherapeutic compounds, it eventually appeared to give an improvement from 70 to 78% survival in patients

with non-metastatic osteosarcoma, which was the best achievement in improving outcome in decades [169;170]. Our finding that macrophages are associated with less metastases now provides a valid biological rationale for the efficacy of this drug. However, concerns regarding the design of the trial has prevented wide-scale clinical adoption of the compound in therapeutic regimens. Additional supportive evidence for the effectiveness of immune-stimulation in osteosarcoma is the use of interferon(IFN)-α as adjuvant therapy, with encouraging results in historical cohorts of Scandinavian osteosarcoma patients [249]. *In vitro* data suggests that the positive effect may involve both immunological (as shown in **chapter 6**) and direct anti-tumor effects [190]. In the recently completed EURAMOS-1 clinical trial this drug was included in one of the randomized arms [278]. Preliminary data suggests no benefit, but follow-up of patients is ongoing [25].

Neither the mechanism of metastasis suppression by intratumoral macrophages in osteosarcoma is clarified, nor the contrast with epithelial tumors. It may be sought in the different flavors of macrophages that are distinguishable by specific markers. M1 are tumor suppressive, M2 support invasion, metastasis and angiogenesis of tumor cells. We assessed the nature of the tumor associated macrophages in osteosarcoma clinical samples using HLA-DRα, associated with M1 macrophages and CD163, a marker to distinguish M2. Surprisingly, both types of macrophages were present in the tumor tissues analyzed (Fig. 7.1). Recent perceptions on good vs. bad macrophages are more nuanced. Macrophages are flexible cells that polarize to a certain direction, but are not destined to stay that way (Fig. 1.5).

To complicate things even more, there was a recent report that macrophage infiltration in another primary bone tumor, Ewing sarcoma, predicts a poor prognosis [78]. Tumor associated macrophages are also associated with a poor prognosis in leiomyosarcoma and gastrointestinal stromal cell tumors [138;266]. The tumor microenvironment is conducive towards the generation of pro-tumor, immunosuppressive and pro-angiogenic M2 macrophages in many epithelial cancers and apparently also in some sarcoma types [208]. On the other hand, a pro-inflammatory tumor microenvironment can skew macrophage polarization towards M1 type macrophages with anti-tumor properties. Similar to epithelial cancers, high macrophage infiltration was associated with increased microvessel density in osteosarcoma, suggesting a similar role for M2 type macrophages in the promotion of angiogenesis. However, in the case of osteosarcoma, the influx of pro-angiogenic macrophages may be similar to a "Trojan horse." Perhaps macrophages are attracted by the tumor to support angiogenesis, but following chemotherapeutic treatment, the release of endogenous danger signals by dying tumor cells causes the macrophages to become polarized toward an M1, anti-tumor phenotype (Fig. 7.2). This proposed mechanism is supported by the fact that the survival benefit of high macrophage infiltration as determined in **chapter 4** was partly dependent on the histological response to chemotherapy.

Coley's toxins were denounced by another famous bone sarcoma expert, the pathologist James Ewing who gave his name to the second aggressive pediatric bone tumor [65]. Ewing was not charmed by the medieval treatment developed by Coley, he was a fervent proponent of radiation therapy, which was effective for many tumors, but not for osteosarcoma.

The relatively good response of sarcomas to immune stimulation and the favorable prognostic effect of tumor associated macrophages as opposed to carcinomas suggests that

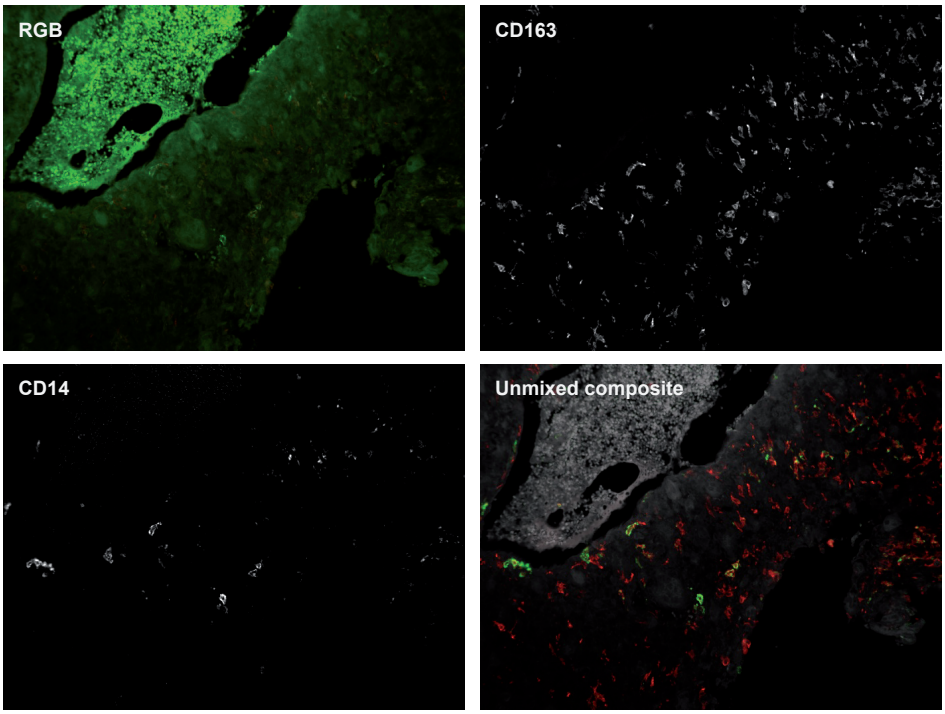


Fig. 7.1 Osteosarcoma samples are infiltrated with CD14 and CD163 single and double positive macrophages. Spectral imaging was used to reduce autofluorescence of osteosarcoma cells. In the composite image, CD14-positive cells are represented in green, CD163-positive cells are represented in red, and CD14/CD163 double positive cells are represented in yellow. Background autofluorescence of tumor cells is represented in gray.

tumor immunology is different for sarcomas. This does not seem attributable to a particular macrophage subtype, but lies in the nature of this tumor type. Few clinical trials have been conducted on immunotherapy in sarcomas. Given our findings that macrophages are associated with less metastases in osteosarcoma, tumor immunotherapy specifically targeted at this tumor type should be evaluated.

Activation of natural killer cells in immunotherapy of osteosarcoma

The ability of natural killer (NK) cells to lyze tumor cells without the need for prior sensitization is an attractive prospect for anticancer immunotherapy [148;186]. In **chapters 5** and **6** we show that osteosarcoma cells are sensitive to lysis by both autologous and allogeneic NK cells. The antitumor activity of NK cells could be further augmented by activation with interleukin(IL)-15 and IFN-α. Importantly, chemotherapy resistant osteosarcoma cells retained their susceptibility to NK cell mediated lysis. Lysis of osteosarcoma cells by NK cells was dependent on Natural Killer Group 2, member D (NKG2D) and DNAX accessory molecule-1 (DNAM-1) but not on CD95. In many cancer types, systemic immunosuppression appears to influence the phenotype and effector capability of immune cells [27;51;52;62]. In contrast, osteosarcoma patient derived NK cells have normal

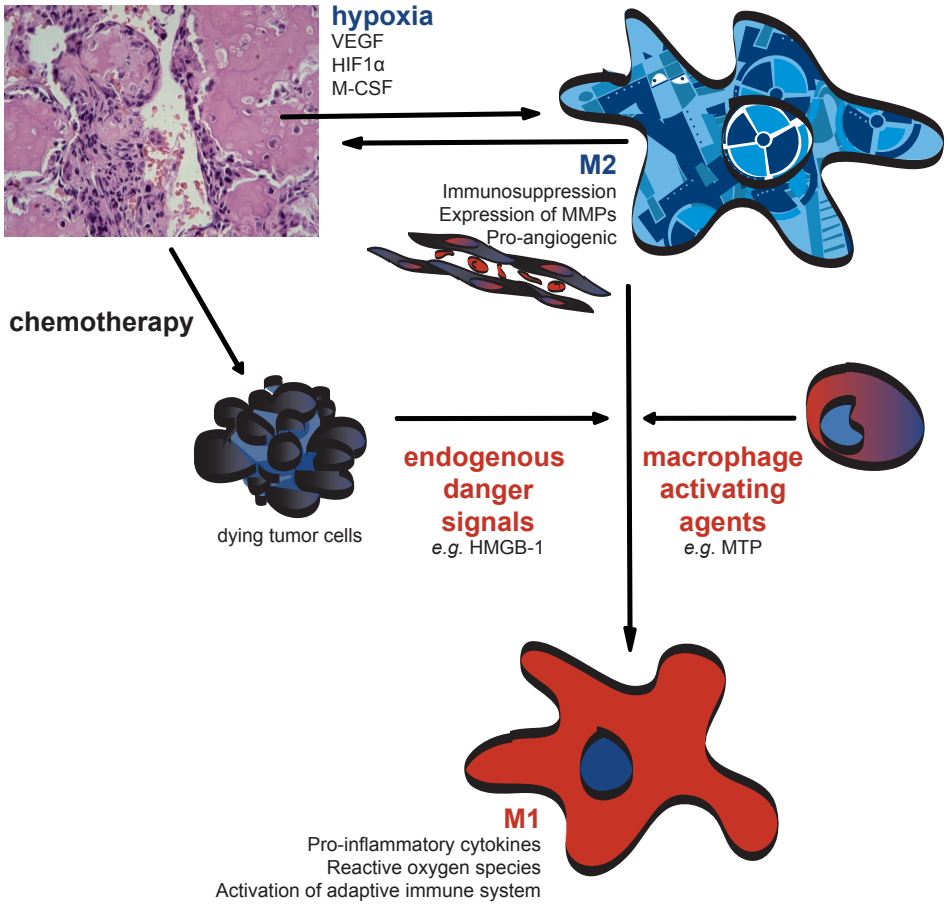


Fig. 7.2 Model depicting the pro-tumor M2 macrophage as a 'Trojan horse' which can polarize towards an anti-tumor M1 type macrophage following activation by danger signals and cytokines.

phenotypic characteristics and unimpaired native cytolytic function (**chapter 5**). In addition, patient derived NK cells can be adequately activated by cytokine treatment with IL-15 (**chapter 5**) or IFN-α (**chapter 6**). Therefore, activation of autologous NK cells (either *in vivo* or *ex vivo*) may be efficacious. However, since patients are treated with lymphodepleting chemotherapy, careful thought needs to be given regarding the optimal timing of adjuvant immunotherapeutic treatment.

A trial in which autologous activated NK cells were infused in melanoma and renal cell cancer patients demonstrated that NK cells persisted in the circulation for days to weeks following adoptive transfer [203]. Disappointingly, no clinical responses were seen, perhaps because of an observed down-regulation of the activating receptor NKG2D *in vivo*. In addition to cytolysis as a result of ligation of activating receptors, NK cells are capable of antibody-dependent cellular cytotoxicity (ADCC). Using the anti-epidermal growth factor receptor (EGFR) antibody cetuximab, osteosarcoma cells are sensitive to ADCC by NK cells [200]. ADCC is not dependent on signaling through the activating

NK cell receptors. Perhaps the combination of the adoptive transfer of autologous NK cells and treatment with humanized monoclonal antibodies will yield the desired clinical results.

Although osteosarcoma cells were sensitive to lysis by autologous NK cells *in vitro*, there is some theoretical benefit of using allogeneic NK cells. NK cells are inhibited in their cytolytic activity when inhibitory killer cell immunoglobulin-like receptors (KIRs) recognize their cognate major histocompatibility complex (MHC) class I ligands (Fig. 1.6) [118]. KIR-ligand mismatch has been shown to contribute to NK cell cytolysis *in vivo,* for example in the setting of stem cell transplantion in acute myeloid leukemia (AML)-patients [173;270]. In line with this data, studies in mice show that KIR-ligand mismatched NK cells facilitate engraftment of hematopoietic transplants, and have increased graft-versus-leukemia efficacy and reduced graft-versus-host disease in comparison to NK cells without such a KIR-ligand mismatch [224]. Similar results were obtained in a murine breast cancer model employing haploidentical bone marrow and spleen transplantation [76]. In osteosarcoma, there is a relatively high level of MHC class I expression (Fig. 5.2, and [262]). Therefore, application of allogeneic NK cells with a KIR-ligand mismatch (for example in the setting of a haploidentical stem cell transplantation) will possibly result in a higher degree of tumor cell lysis than strategies aimed at the activation of autologous NK cells.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Despite multi-agent chemotherapy and wide margin surgery, high-grade osteosarcoma has a poor prognosis. In this thesis, data demonstrating a role for cells of the innate immune system in controlling and possibly treating high-grade osteosarcoma are presented. Future studies should be aimed at a better understanding of the interaction between tumor and host. Tumor cells may influence migration, differentiation, polarization and activation of innate immune cells. Conversely, immune cells can have direct or indirect effects on viability, motility, invasion and migration of tumor cells. For example, immune cells can express cytokines and chemokines -such as CXCL12- which can bind to the receptors – in this case CXCR4- on tumor cells, with a potential to influence tumor cell viability and motility. There is substantial interconnectivity between these various aspects of tumor-host interactions, which also involve other cells, such as endothelial cells and stromal cells.

As was shown in **chapter 4**, infiltration of macrophages in osteosarcoma is associated with a reduced risk of metastatic disease, but the exact biological mechanism in which macrophages inhibit (metastasis of) osteosarcoma tumor cells remains unclear. Both classically activated M1 type macrophages as well as alternatively activated M2 type macrophages are present in osteosarcoma. Using an *in vitro* co-culture system, the effect of osteosarcoma cells on the differentiation and polarization of monocytes and macrophages can be studied. In addition, cytotoxicity experiments can be performed to study if macrophages are able to lyse osteosarcoma cells and if this is dependent on the activation and polarization status (M1 *vs.* M2) of the effector cells. Upregulation of the SIRPα-ligand CD47 on myeloid leukemia cells resulted in reduced phagocytosis by macrophages, possibly contributing to immune evasion by the tumor [109]. Although the interaction between CD47 and SIRPα was not studied in this thesis, in **chapter 4**, several macrophage genes associated with phagocytosis were shown to be

upregulated in osteosarcoma patients with good prognosis. Perhaps specific blocking of CD47-SIRPα interaction augments macrophage-mediated osteosarcoma cell phagocytosis.

A promising tool to study tumor-host interactions are xenograft models [182]. Freshly isolated tumor cells obtained from diagnostic biopsies can be transplanted in immunodeficient mice. If corresponding peripheral blood mononuclear cells (PBMCs) are available from these patients, these can be used to reconstitute the immune system of the recipient. This will allow for detailed study of tumor-immune cell interaction in an 'autologous' setting. Tumor growth can be monitored in the presence or absence of specific immune cell subsets. Using such an 'autologous xenograft model', several fundamental questions regarding the interaction between monocytes/macrophages and osteosarcoma cells can be answered. Do monocytes/macrophages influence growth of osteosarcoma xenografts? If so, is this effect dependent on the presence of other immune cell subsets such as T cells, implicating a possible role of the adaptive immune system in tumor control? Does treatment with macrophage activating agents influence macrophage polarization and tumor outgrowth in this model? Using a humanized antibody, is there evidence of ADCC by monocytes/macrophages and/or NK cells *in vivo?* Is there an effect of monocytes/macrophages on angiogenesis in osteosarcoma and is this dependent on polarization status of the macrophages?

In **chapters 5 and 6**, pre-clinical data regarding the efficacy of NK cells in the treatment of osteosarcoma are presented. As was discussed in paragraph 7.2.2, there is a theoretical advantage of the adoptive transfer of KIR-ligand mismatched allogeneic NK cells as opposed to the activation of autologous NK cells. Additional *in vitro* cytolytic experiments can determine if KIR-ligand mismatch also contributes to increased NK cell mediated cytotoxic activity in osteosarcoma. Xenograft models using immunodeficient mice with human NK cell reconstitution can be used to study efficacy of NK cells and NK cell activating agents in osteosarcoma lysis *in vivo*. Another potentially efficacious approach in augmenting NK cell mediated cytotoxicity is blocking the effect of non-KIR co-inhibitory receptors. For example, NK cells can express the inhibitory receptor PD-1 [289]. Perhaps blocking of this receptor might result in a better lysis of PD-1 ligand expressing tumor cells.

A hurdle in successful application of NK cell activation or adoptive transfer in the immunotherapy is the infiltration of sufficient numbers of activated NK cells in the tumor. Zebrafish or murine xenografts with live cell imaging of labeled immune cells can be used to study migration of immune cells towards the tumor site.

In addition to the preclinical studies, the results as presented in this thesis justify translation to clinical trials. Treatment with mifamurtide -a macrophage activating agent-, yielded promising clinical results [169;170], but it remains unknown which patient group would benefit most from treatment with macrophage activating agents. Perhaps treatment with a macrophage activating agent is only beneficial in patients that already have relatively large numbers of macrophages present in the tumor. Alternatively, activation of monocytes circulating in the peripheral blood could also result in enhanced intratumoral infiltration and subsequent antitumor activity of intratumoral macrophages and will thus (also) benefit patients who present with low numbers of macrophages in the primary tumor. To better understand

what is happening *in situ* during treatment with a monocyte/macrophage activating agent, the migration and activation status of monocytes/macrophages before, during and after treatment needs to be monitored. Patients with bilateral metastatic disease, eligible for multi-step surgery, could undergo resection of metastatic lesions in one lung, followed by several weeks of treatment with a macrophage activating agent such as mifamurtide. Subsequently, the metastases in the contralateral lung could be resected. In this way, the numbers and activation status of macrophages infiltrating the tumor can be determined, both before and after treatment with a macrophage activating agent. PBMCs and plasma can be collected at several time-points to monitor the activation status of monocytes and cytokine levels during this process. This will yield important information regarding the mechanism of action of macrophages in inhibiting metastasis in osteosarcoma.

The safety and efficacy of NK-cell based immunotherapy in osteosarcoma should be addressed in a phase I/II clinical trial. A possible strategy could be the adoptive transfer of cytokine-activated donor NK cells in an allogeneic (especially haploidentical) stem cell transplantation setting. In this scenario, KIR-ligand mismatch will hopefully result in maximal efficacy. Alternatively, autologous NK cells can be activated by the administration of cytokines, either *in vivo* or *ex vivo*. IFN-signaling was unimpaired in PBMCs of osteosarcoma patients and osteosarcoma cells were efficiently killed by IFN- α activated NK cells (**chapter 6**). Preliminary results of the EURAMOS-1 trial however, did not show a clinical benefit of monotherapy with IFN-α in osteosarcoma patients with good histological response to neo-adjuvant chemotherapy [25]. The *in vivo* activation of NK cells might not be as effective as the *ex vivo* activation, possibly due to being able to achieve higher concentrations *in vitro*. Perhaps the adoptive transfer of cytokine-activated NK cells is more efficacious than the administration of cytokines to patients in an attempt to activate immune cells *in vivo*.

In conclusion, the activation of innate immune cells such as macrophages and NK cells is a promising new adjuvant treatment strategy to treat patients with high-grade osteosarcoma. Using xenograft models, the interactions between tumor and host can be examined in more detail. Further studies should be aimed at the translation of pre-clinical data towards clinical trials exploiting the potential of the innate immune system in controlling high-grade osteosarcoma.

8.

Nederlandse samenvatting

Hooggradig osteosarcoom is een agressieve vorm van botkanker die voornamelijk adolescenten en jong volwassenen treft. Het is een zeldzame tumor, met afhankelijk van de leeftijd een incidentie tussen de 1.7 en 8.4 per miljoen. Ondanks behandeling met chemotherapie en operatieve verwijdering van de tumor, overlijdt dertig tot veertig procent van de patiënten aan de ziekte, meestal ten gevolge van uitzaaiingen naar de longen. In dit proefschrift werden factoren gerelateerd aan de etiologie en prognose van het hooggradige osteosarcoom onderzocht (**hoofdstuk 2** en **3**). Daarnaast werd de interactie tussen specifieke cellen van het aangeboren afweersysteem en osteosarcoom onderzocht (**hoofdstuk 4**, **5** en **6**). Gezamenlijk zullen deze studies hopelijk leiden tot de ontwikkeling van therapieën die de cellen van het aangeboren afweersysteem activeren en in staat stellen de groei van tumorcellen in te perken, dan wel de tumorcellen direct of indirect te doden.

In sommige gevallen van osteosarcoom is er sprake van een genetische predispositie, bijvoorbeeld zoals in het kader van het Li-Fraumeni syndroom (ten gevolge van *TP53* mutaties) of het erfelijke retinoblastoom syndroom (ten gevolge van *RB1* mutaties). In de meeste gevallen is er echter geen erfelijke oorzaak aan te wijzen. Slechts zelden is er in het bot een pre-maligne afwijking aan te wijzen. Er zijn een aantal aanwijzingen dat mesenchymale stroma cellen (MSCs) of vroege osteoblast-precursorcellen de voorloper cellen zijn van osteosarcoomcellen. Ten eerste, vindt er in de groeischijven ten tijde van de pubertaire groeispurt een snelle celdeling en differentiatie van MSCs plaats. Het is op deze anatomische lokatie dat osteosarcoom het meest frequent ontstaat. Ten tweede is gebleken dat muizen MSCs die langdurig *in vitro* gekweekt zijn, zich kunnen ontwikkelen tot , osteosarcoom-achtige tumorcellen. Ook MSCs van apen (*Macacus cynomolgus*) ondergaan een dergelijke transformatie naar tumor-achtige cellen bij langdurig kweken. In **hoofdstuk 2** wordt beschreven dat bij het langdurig kweken van MSCs van osteosarcoompatiënten, een dergelijke kwaadaardige ontaarding niet plaatsvond. Ondanks bijna twee jaar continue kweek, werd in geen van de MSC kweken de plotselinge toename van proliferatie gezien welke karakteristiek is voor *in vitro* maligne ontaarding. Wel ontstonden er, zowel in MSCs van gezonde donoren als in MSCs van osteosarcoompatiënten, gedurende het kweken binucleaire cellen. Bij genexpressieanalyse van lage passages MSCs bleek bij MSCs afkomstig van osteosarcoompatiënten een lagere expressie van "hematopoietic cell specific Lyn substrate 1" (*HCLS1*) dan MSCs afkomstig van gezonde donoren. Het eiwit afkomstig van *HCLS1* staat bekend om zijn rol bij B-cel receptor signalering en myelopoiese. Of verminderde expressie van dit gen in MSCs een rol speelt bij de ontwikkeling tot osteosarcoom is nog onbekend.

Behalve de differentiële expressie van *HCLS1* waren de MSCs van osteosarcoompatiënten en gezonde donoren zeer overeenkomstig. Er zijn verschillende mogelijke oorzaken voor het feit dat er zo weinig verschillen tussen MSCs van osteosarcoompatiënten en gezonde donoren waren. Het zou zo kunnen zijn dat de hypothese dat het osteosarcoom uit MSCs ontstaat, niet juist is. Daarnaast zou het kunnen zijn dat op de plek waar de MSCs voor deze studie geoogst zijn (de bovenrand van het heupbeen) de cellen niet bovenmatig gevoelig voor maligne ontaarding zijn, terwijl voorlopercellen op andere plaatsen (*in casu* aan het uiteinde van de lange pijpbeenderen waar de tumoren zich bevinden) dit wel zijn. Deze lokatiespecifieke gevoeligheid voor tumorontwikkeling kan aan intrinsieke verschillen in de voorlopercellen liggen (bijvoorbeeld

bij somatisch mozaïcisme), of aan specifieke omgevingsfactoren in de buurt van de groeischijf (endocriene en paracriene signalering). Een andere mogelijke verklaring voor het uitblijven van oncogene transformatie tijdens langdurig kweken, is dat dit onvoldoende is om kwaadaardige ontaarding plaats te laten vinden. Er wordt algemeen aangenomen dat er meerdere 'hits' nodig zijn om een gezonde cel tot een tumorcel te laten verworden. Mogelijk dat lokaal aanwezige groeifactoren of additionele genetische of functionele veranderingen zoals verlies van celcyclus controle noodzakelijk zijn voordat transformatie optreedt. Ook indien de tumorontwikkeling niet volgens een stapsgewijze opeenstapeling van genetische fouten ontstaat, maar er een catastrofale chromosomale gebeurtenis genaamd 'chromotripsis' optreedt, moet deze waarschijnlijk in een voor kwaadaardige ontaarding gevoelige genetische of omgevingsachtergrond plaatsvinden.

In **hoofdstuk 3** wordt beschreven welke factoren van prognostisch belang zijn voor de overleving van osteosarcoompatiënten met longmetastasen. Hieruit bleek, dat het aantal (histologisch vitale) longmetastasen en het mannelijke geslacht geassocieerd waren met een hogere kans op overlijden. In **hoofdstuk 4** werd middels een genoombrede genexpressie analyse in een relatief groot cohort van osteosarcoompatiënten aangetoond dat indien er een hoge expressie van macrofaag-geassocieerde genen in tumorbiopten aanwezig was, dit geassocieerd was met een lager risico voor gemetastaseerde ziekte. De resultaten beschreven in deze hoofdstukken impliceren dat patiënten met morfologisch vitale en/of een groot aantal metastasen baat zouden kunnen hebben bij immunotherapeutische strategieën die migratie naar en activatie van monocyten en macrofagen in osteosarcoom stimuleren.

De tumorgroei-bevorderende rol van tumor geassocieerde macrofagen in carcinomen is welbekend. Epitheliale tumoren met grote aantallen infiltrerende macrofagen hebben een slechtere prognose dan tumoren met weinig infiltrerende cellen, wat gerelateerd is aan de belangrijke rol die macrofagen spelen in de initiatie van angiogenese van deze tumoren. Macrofagen zijn zeer plastische cellen die, afhankelijk van hun omgeving en de activerende signalen die ze ontvangen, een tumor bevorderende of tumor inhiberende rol aan kunnen nemen. Cytokines zoals interferon (IFN)-γ en bacteriële cel(wand)producten zoals muramyl-tri-peptide (MTP) kunnen macrofagen 'klassiek' activeren. Deze 'klassiek geactiveerde' M1-macrofagen brengen pro-inflammatoire cytokines zoals interleukine (IL)-1, IL-6 en IL-12 tot expressie en kunnen tumorcellen direct doden door fagocytose, zuurstofradicalen en cytokine-geïnduceerde cytotoxiciteit. Daarnaast kunnen zij NK en T cellen rekruteren en activeren en zo ook indirect voor tumorceldood zorgen. Door IL-4 en IL-13 'alternatief geactiveerde' M2 macrofagen zijn belangrijk in weefselhomeostase en bevorderen wondgenezing en angiogenese. Het zijn deze M2 karakteristieken die vaak in verband gebracht worden met de 'pro-tumor' kwaliteiten van tumor geassocieerde macrofagen.

Zoals beschreven in **hoofdstuk 4**, waren een aantal genen die hoog tot expressie kwamen in biopten afkomstig van patiënten zonder metastasen, specifieke macrofaagmarkers zoals *CD14* en *MSR1*. Daarnaast bleek een groot aantal van de genen die hoog tot expressie kwamen in deze prognostisch gunstigere groep geassocieerd met macrofaagfuncties zoals antigeen verwerking en presentatie. Middels aanvullende immunohistochemische en genexpressie studies werd aangetoond dat de macrofaag geassocieerde genen inderdaad door macrofagen en niet door

tumorcellen tot expressie gebracht werden. In aanvullende cohorten werd bevestigd dat een hogere infiltratie met macrofagen geassocieerd was met een betere overleving.

William B. Coley was een chirurg die bottumoren behandelde. In 1891 voerde hij de eerste succesvolle immunotherapeutische behandelingen uit door bij patiënten met inoperabele tumoren een mengsel van bacteriële toxines in de tumor te spuiten. De grootste successen met deze zogeheten 'Coley's toxines' werden bij patiënten met tumoren van mesodermale origine, zoals osteosarcoom, geboekt. Ook meer recent zijn er aanwijzingen dat de klassieke activatie van macrofagen middels bacteriële celproducten tot een betere overleving van osteosarcoompatiënten leidt. Osteosarcoompatiënten met een postoperatieve wondinfectie hebben op de langere termijn een betere overleving, hetgeen bij muizenstudies afhankelijk van NK cellen en monocyten/macrofagen was. Een klinische trial waarbij liposomaal MTP (ook bekend onder de naam mifamurtide) werd toegevoegd aan de standaard adjuvante chemotherapeutische behandeling van osteosarcoompatiënten resulteerde in een significante verbetering in de zesjaarsoverleving van 70% naar 78%.

Het mechanisme waarmee macrofagen de metastasering in osteosarcoom direct of indirect inhiberen is nog niet opgehelderd. Zowel macrofagen met een M1 fenotype (met een hoge expressie van HLA-DRα) als macrofagen met een M2 fenotype (met een hoge expressie van CD163) waren aanwezig in osteosarcomen. Het aantal macrofagen in de tumor was positief gecorreleerd met zowel een betere histologische respons op chemotherapie, als met een groter aantal vaten in de tumor, een maat voor angiogenese. Mogelijk is de influx van macrofagen in osteosarcoom een 'Trojaans paard', waarbij ze als M2-type macrofagen de tumor ondersteunen door expressie van matrix metalloproteinasen (MMPs) en het beïnvloeden van de angiogenese. Ten gevolge van endogene (door immunogene celdood) of exogene (door de toediening van bijvoorbeeld MTP) 'danger' signalen kan vervolgens een verandering van fenotype richting een anti-tumor M1 macrofaag plaatsvinden en wordt de aanvankelijke vriend, de vijand van de tumorcellen (Fig. 7.2).

In toekomstige studies dient het effect van osteosarcoomcellen op de differentiatie en polarisatie van monocyten en macrofagen en *vice versa* onderzocht te worden, zowel middels *in vitro* modellen als in *in vivo* xenograft modellen. Toekomstige klinische trials moeten zijn ontworpen om niet alleen de effectiviteit van macrofaag activerende agentia zoals mifamurtide te onderzoeken, maar ook de onderliggende mechanismen te bestuderen. Het onderzoeken van biologisch materiaal (perifere bloedcellen, serum en tumormateriaal) voorafgaand, gedurende en na de klinische trial is daarbij essentieel. Alleen dan kan antwoord worden gegeven op een aantal essentiële vragen. Ten eerste, maakt het aantal macrofagen dat bij aanvang van de behandeling aanwezig is in de tumor uit voor de effectiviteit van therapeutische macrofaagactivatie? Ten tweede, wat is de rol van chemotherapie en immunogene celdood van OS in de activatie van macrofagen? Ten derde, is er een rol voor de secundaire activatie van het adaptieve immuunsysteem in de effectiviteit van deze op het aangeboren immuunsysteem gerichte therapieën?

Natural killer (NK) cellen herkennen en doden virusgeïnfecteerde en getransformeerde cellen wanneer de balans tussen signalen afkomstig van activerende en inhiberende receptoren naar activatie overslaat (Fig. 1.6). Osteosarcoomcellen bleken in hoge mate gevoelig voor lysis door NK cellen (**hoofdstuk 5** en **6**), wat nog verder verbeterd kon worden door de NK cellen met cytokines

zoals IL-15 en IFN-α te activeren. Belangrijk was, dat ook chemotherapie-resistente cellen gevoelig waren voor lysis door NK cellen. NK cellen van osteosarcoompatiënten hadden een fenotype en een lytische capaciteit die vergelijkbaar was met NK cellen van gezonde donoren. Ook in een autologe setting was er enige cytolytische activiteit van NK cellen tegen de eigen osteosarcoomcellen, met een goede verbetering van de cytolytische activiteit na voorbehandeling met IL-15 of IFN- α. Deze resultaten impliceren dat zowel autologe als allogene geactiveerde NK cellen (hetzij *in vivo* dan wel *ex vivo*) een mogelijke effectieve therapie zou kunnen zijn voor osteosarcoompatiënten. Er is echter een theoretisch voordeel van het gebruiken van allogene NK cellen. NK cellen worden in hun cytolytische activiteit geïnhibeerd wanneer 'killer cell immunoglobulin-like' receptoren (KIRs) aan de bijbehorende 'major histocompatibility complex' (MHC) klasse 1 liganden binden. In een allogene setting, kan er sprake zijn van een 'mismatch' tussen de KIRs en de MHC klasse 1 liganden, wat resulteert een vermindering van de inhiberende signalering en theoretisch een grotere effectiviteit van NK cel gebaseerde immunotherapie. Gezien de relatief hoge expressie van MHC klasse 1 op osteosarcoomcellen, zou dit een belangrijke overweging kunnen zijn in het ontwerpen van toekomstige klinische trials.

Een nog onbeantwoorde vraag is, of er *in vivo* voldoende migratie van NK cellen optreedt naar de tumorcellen om tot klinisch relevante tumorcellysis te leiden. Mogelijk dat xenograft modellen met het in beeld brengen van geïnfundeerde, gelabelde effector cellen hier een antwoord op kan geven. Ook kan in preklinische diermodellen onderzocht worden wat de toegevoegde rol zou kunnen zijn voor 'antibody dependent cellular toxicity' (ADCC). Bij ADCC herkent een (gehumaniseerd) antilichaam de tumorcel en bindt vervolgens via het Fc-domein aan speciale antilichaamreceptoren (Fc-receptoren) op monocyten of NK cellen. Dit concept is bestudeerd in *in vitro* studies waarbij bleek dat middels behandeling met het anti-EGFR antilichaam cetuximab op deze wijze een toename van de activiteit van NK cellen richting osteosarcoomcellen kon worden bereikt.

De veiligheid en effectiviteit van NK cel gebaseerde immunotherapeutische strategieën dient in fase I/II klinische trials verder onderzocht te worden. Een mogelijke strategie is de infusie van cytokine-geactiveerde donor NK cellen in een allogene (met name haplo-identieke) stamceltransplantatie-setting. Op deze wijze kan maximaal gebruik worden gemaakt van de KIR-ligand mismatch tussen donor en ontvanger en zal er hopelijk maximale anti-tumor effectiviteit worden behaald.

Nieuwe behandelingen voor patiënten met hooggradig osteosarcoom zijn hard nodig, zeker indien er sprake is van gemetastaseerd ziekte. De afgelopen twintig jaar is er nauwelijks verbetering in de overleving van deze vaak jonge patiëntengroep bereikt, ondanks hoge dosis chemotherapie en agressieve chirurgie. Het toepassen van immunotherapie is een veelbelovende strategie, met name in een multimodale setting gecombineerd met chemotherapie. Hierbij kan optimaal gebruik kan worden gemaakt van de immunogene celdood die door specifieke chemotherapie kan worden geïnduceerd, waarna (geactiveerde) immuuncellen verdere immunosurveillance en antitumor cytolysis kunnen zorgen. Vanwege de zeldzaamheid van deze tumor en de benodigde expertise in zowel behandeling als biological monitoring, dienen nieuwe therapieën vanuit expertise centra en idealiter in internationaal samenwerkingsverband te worden onderzocht.

&.

Addendum

REFERENCES

1. **Eur**opean and **Am**erican **O**steosarcoma **S**tudy Group. http://www.ctu.mrc.ac.uk/euramos/
2. WHO Classification of Tumours of Soft Tissue and Bone. Lyon: International Agency for Research on Cancer, 2013.
3. Addison CL, Daniel TO, Burdick MD, Liu H, Ehlert JE, Xue YY, Buechi L, Walz A, Richmond A, Strieter RM. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. J Immunol 2000;165:5269-5277.
4. Aksnes LH, Hall KS, Folleraas G, Stenwig AE, Bjerkehagen B, Taksdal I, Winderen M, Bruland OS, Saeter G. Management of high-grade bone sarcomas over two decades: The Norwegian Radium Hospital experience. Acta Oncologica 2006;45:38-46.
5. Allavena P, Mantovani A. Immunology in the clinic review series; focus on cancer: tumour-associated macrophages: undisputed stars of the inflammatory tumour microenvironment. Clin Exp Immunol 2012;167:195-205.
6. Ancuta P, Liu KY, Misra V, Wacleche VS, Gosselin A, Zhou XB, Gabuzda D. Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16+and CD16-monocyte subsets. Bmc Genomics 2009;10.
7. Anguille S, Lion E, Willemen Y, Van Tendeloo VF, Berneman ZN, Smits EL. Interferon-alpha in acute myeloid leukemia: an old drug revisited. Leukemia 2011;25:739-748.
8. Anninga JK, Gelderblom H, Fiocco M, Kroep JR, Taminiau AH, Hogendoorn PCW, Egeler RM. Chemotherapeutic adjuvant treatment for osteosarcoma: where do we stand? Eur J Cancer 2011;47:2431-2445.
9. Aoki N, Kimura Y, Kimura S, Nagato T, Azumi M, Kobayashi H, Sato K, Tateno M. Expression and functional role of MDL-1 (CLEC5A) in mouse myeloid lineage cells. Journal of Leukocyte Biology 2009;85:508-517.
10. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, Andre F, Delaloge S, Tursz T, Kroemer G, Zitvogel L. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nature Medicine 2007;13:1050-1059.
11. Athanasou NA, Quinn J. Immunophenotypic Differences Between Osteoclasts and Macrophage Polykaryons - Immunohistological Distinction and Implications for Osteoclast Ontogeny and Function. Journal of Clinical Pathology 1990;43:997-1003.
12. Bacci G, Briccoli A, Longhi A, Ferrari S, Mercuri M, Faggioli F, Versari M, Picci P. Treatment and outcome of recurrent osteosarcoma: Experience at Rizzoli in 235 patients initially treated with neoadjuvant chemotherapy. Acta Oncologica 2005;44:748-755.
13. Bacci G, Longhi A, Versari M, Mercuri M, Briccoli A, Picci P. Prognostic factors for osteosarcoma of the extremity treated with neoadjuvant chemotherapy - 15-year experience in 789 patients treated at a single institution. Cancer 2006;106:1154-1161.
14. Bacci G, Rocca M, Salone M, Balladelli A, Ferrari S, Palmerini E, Forni C, Briccoli A. High Grade Osteosarcoma of the Extremities With Lung Metastases at Presentation: Treatment With Neoadjuvant Chemotherapy and Simultaneous Resection of Primary and Metastatic Lesions. Journal of Surgical Oncology 2008;98:415-420.
15. Ball LM, Bernardo ME, Roelofs H, Lankester A, Cometa A, Egeler RM, Locatelli F, Fibbe WE. Cotransplantation of ex vivo-expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. Blood 2007;110:2764-2767.
16. Bauer S, Pigisch S, Hangel D, Kaufmann A, Hamm S. Recognition of nucleic acid and nucleic acid analogs by Toll-like receptors 7, 8 and 9. Immunobiology 2008;213:315-328.
17. Begum NA, Tsuji S, Nomura R, Shida K, Azuma I, Hayashi A, Matsumoto M, Seya T, Toyoshima K. Human MD-1 homologue is a BCG-regulated gene product in monocytes: Its identification by differential display. Biochemical and Biophysical Research Communications 1999;256:325-329.
18. Ben-David U, Mayshar Y, Benvenisty N. Large-scale analysis reveals acquisition of lineage-specific chromosomal aberrations in human adult stem cells. Cell Stem Cell 2011;9:97-102.
19. Berger M, Muraro M, Fagioli F, Ferrari S. Osteosarcoma Derived from Donor Stem Cells Carrying the Norrie's Disease Gene. New England Journal of Medicine 2008;359:2502-2504.
20. Berghuis D, de Hooge ASK, Santos SJ, Horst D, Wertz EJ, van Eggermond MC, van den Elsen PJ, Taminiau AHM, Ottaviano L, Schaefer KL, Dirksen U, Hooijberg E, Mulder A, Melief CJM, Egeler RM, Schilham MW,

Jordanova ES, Hogendoorn PCW, Lankester AC. Reduced human leukocyte antigen expression in advanced-stage Ewing sarcoma: implications for immune recognition. J Pathol 2009;218:222-231.

21. Bernardo ME, Emons JAM, Karperien M, Nauta AJ, Willemze R, Roelofs H, Romeo S, Marchini A, Rappold GA, Vukicevic S, Locatelli F, Fibbe WE. Human mesenchymal stem cells derived from bone marrow display a better chondrogenic differentiation compared with other sources. Connective Tissue Research 2007;48:132-140.
22. Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, Montagna D, Maccario R, Villa R, Daidone MG, Zuffardi O, Locatelli F. Human bone marrow-derived mesenchymal stem cells do not undergo transformation after long-term In vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Research 2007;67:9142-9149.
23. Bielack SS, Kempf-Bielack B, Branscheid D, Carrle D, Friedel G, Helmke K, Kevric M, Jundt G, Kuhne T, Maas R, Schwarz R, Zoubek A, Jurgens H. Second and subsequent recurrences of osteosarcoma: presentation, treatment, and outcomes of 249 consecutive cooperative osteosarcoma study group patients. J Clin Oncol 2009;27:557-565.
24. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, Zoubek A, Jurgens H, Winkler K. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: An analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 2002;20:776-790.
25. Bielack SS, Smeland S, Whelan J, Marina N, Hook J, Jovic G, Kramer MD, Butterfass-Bahloul T, Kuhne T, Eriksson M, Teot L, Gelderblom H, Kager L, Hall KS, Gorlick R, Randall RL, Hogendoorn PCW, Calaminus G, Sydes MR, Bernstein ML. MAP plus maintenance pegylated interferon a-2b (MAPIfn) versus MAP alone in patients with resectable high-grade osteosarcoma and good histologic response to preoperative MAP: First results of the EURAMOS-1 "good response" randomization. J Clin Oncol (Meeting Abstracts) 2013;31:suppl; abstr LBA10504.
26. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, Cantoni C, Grassi J, Marcenaro S, Reymond N, Vitale M, Moretta L, Lopez M, Moretta A. Identification of PVR (CD155) and nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. J Exp Med 2003;198:557-567.
27. Boudny V, Dusek L, Adamkova L, Chumchalova J, Kocak I, Fait V, Lauerova L, Krejci E, Kovarik J. Lack of STAT 1 phosphorylation at TYR 701 by IFNgamma correlates with disease outcome in melanoma patients. Neoplasma 2005;52:330-337.
28. Bowdish DM, Gordon S. Conserved domains of the class A scavenger receptors: evolution and function. Immunol Rev 2009;227:19-31.
29. Briccoli A, Rocca M, Salone M, Bacci G, Ferrari S, Balladelli A, Mercuri M. Resection of recurrent pulmonary metastases in patients with osteosarcoma. Cancer 2005;104:1721-1725.
30. Bridge JA, Nelson M, McComb E, McGuire MH, Rosenthal H, Vergara G, Maale GE, Spanier S, Neff JR. Cytogenetic findings in 73 osteosarcoma specimens and a review of the literature. Cancer Genet Cytogenet 1997;95:74-87.
31. Brosjo O, Bauer HC, Brostrom LA, Nilsonne U, Nilsson OS, Reinholt FP, Strander H, Tribukait B. Influence of human alpha-interferon on four human osteosarcoma xenografts in nude mice. Cancer Res 1985;45:5598-5602.
32. Buddingh EP, Anninga JK, Versteegh MI, Taminiau AH, Egeler RM, van Rijswijk CS, Hogendoorn PCW, Lankester AC, Gelderblom H. Prognostic factors in pulmonary metastasized high-grade osteosarcoma. Pediatr Blood Cancer 2010;54:216-221.
33. Buddingh EP, Kuijjer ML, Duim RA, Burger H, Agelopoulos K, Myklebost O, Serra M, Mertens F, Hogendoorn PCW, Lankester AC, Cleton-Jansen AM. Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating agents. Clin Cancer Res 2011;17:2110-2119.
34. Buddingh EP, Schilham MW, Ruslan SE, Berghuis D, Szuhai K, Suurmond J, Taminiau AH, Gelderblom H, Egeler RM, Serra M, Hogendoorn PCW, Lankester AC. Chemotherapy-resistant osteosarcoma is highly susceptible to IL-15-activated allogeneic and autologous NK cells. Cancer Immunol Immunother 2011;60:575-586.
35. Buhtoiarov IN, Lum HD, Berke G, Sondel PM, Rakhmilevich AL. Synergistic activation of macrophages via CD40 and TLR9 results in T cell independent antitumor effects. Journal of Immunology 2006;176:309-318.
36. Bystrom J, Tenno T, Hakansson L, Amin K, Trulson A, Hogbom E, Venge P. Monocytes, but not macrophages, produce the eosinophil cationic protein. Apmis 2001;109:507-516.
37. Carim-Todd L, Escarceller M, Estivill X, Sumoy L. Cloning of the novel gene TM6SF1 reveals conservation of clusters of paralogous genes be tween human chromosomes 15q24 -> q26 and 19p13.3 -> p12. Cytogenetics and Cell Genetics 2000;90:255-260.

38. Chang CC, Ferrone S. Immune selective pressure and HLA class I antigen defects in malignant lesions. Cancer Immunol Immunother 2007;56:227-236.
39. Chen ST, Lin YL, Huang MT, Wu MF, Cheng SC, Lei HY, Lee CK, Chiou TW, Wong CH, Hsieh SL. CLEC5A is critical for dengue-virus-induced lethal disease. Nature 2008;453:672-U12.
40. Chiang KP, Niessen S, Saghatelian A, Cravatt BF. An enzyme that regulates ether lipid signaling pathways in cancer annotated by multidimensional profiling. Chemistry & Biology 2006;13:1041-1050.
41. Cho D, Shook DR, Shimasaki N, Chang YH, Fujisaki H, Campana D. Cytotoxicity of activated natural killer cells against pediatric solid tumors. Clin Cancer Res 2010;16:3901-3909.
42. Chou AJ, Merola PR, Wexler LH, Gorlick RG, Vyas YM, Healey JH, LaQuaglia MP, Huvos AG, Meyers PA. Treatment of osteosarcoma at first recurrence after contemporary therapy - The Memorial Sloan-Kettering Cancer Center Experience. Cancer 2005;104:2214-2221.
43. Chowdhry M, Hughes C, Grimer RJ, Sumathi V, Wilson S, Jeys L. Bone sarcomas arising in patients with neurofibromatosis type 1. J Bone Joint Surg Br 2009;91:1223-1226.
44. Chowdhury D, Lieberman J. Death by a thousand cuts: Granzyme pathways of programmed cell death. Annu Rev Immunol 2008;26:389-420.
45. Chung S, Furihata M, Tamura K, Uemura M, Daigo Y, Nasu Y, Miki T, Shuin T, Fujioka T, Nakamura Y, Nakagawa H. Overexpressing PKIB in prostate cancer promotes its aggressiveness by linking between PKA and Akt pathways. Oncogene 2009;28:2849-2859.
46. Cipriani P, Carubbi F, Liakouli V, Marrelli A, Perricone C, Perricone R, Alesse E, Giacomelli R. Stem cells in autoimmune diseases: Implications for pathogenesis and future trends in therapy. Autoimmun Rev 2012.
47. Cleton-Jansen AM, Anninga JK, Briaire-de B, I, Romeo S, Oosting J, Egeler RM, Gelderblom H, Taminiau AH, Hogendoorn PCW. Profiling of high-grade central osteosarcoma and its putative progenitor cells identifies tumourigenic pathways. Br J Cancer 2009;101:2064.
48. Coffelt SB, Hughes R, Lewis CE. Tumor-associated macrophages: effectors of angiogenesis and tumor progression. Biochim Biophys Acta 2009;1796:11-18.
49. Coley WB. The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. Clin Orthop Relat Res 1991;3-11.
50. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell 2000;103:481-490.
51. Critchley-Thorne RJ, Simons DL, Yan N, Miyahira AK, Dirbas FM, Johnson DL, Swetter SM, Carlson RW, Fisher GA, Koong A, Holmes S, Lee PP. Impaired interferon signaling is a common immune defect in human cancer. Proceedings of the National Academy of Sciences of the United States of America 2009;106:9010-9015.
52. Critchley-Thorne RJ, Yan N, Nacu S, Weber J, Holmes SP, Lee PP. Down-regulation of the interferon signaling pathway in T lymphocytes from patients with metastatic melanoma. PLoS Med 2007;4:e176.
53. Crompton BD, Goldsby RE, Weinberg VK, Feren R, O'Donnell RJ, Ablin AR. Survival after recurrence of osteosarcoma: A 20-year experience at a single institution. Pediatric Blood & Cancer 2006;47:255-259.
54. Darnell JE, Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994;264:1415-1421.
55. De Gassart A, Camosseto V, Thibodeau J, Ceppi M, Catalan N, Pierre P, Gatti E. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. Proceedings of the National Academy of Sciences of the United States of America 2008;105:3491-3496.
56. de la Fuente R, Bernad A, Garcia-Castro J, Martin MC, Cigudosa JC. Retraction: Spontaneous human adult stem cell transformation. Cancer Res 2010;70:6682.
57. Dohi O, Hatori M, Suzuki T, Ono K, Hosaka M, Akahira JI, Miki Y, Nagasaki S, Itoi E, Sasano H. Sex steroid receptors expression and hormone-induced cell proliferation in human osteosarcoma. Cancer Science 2008;99:518-523.
58. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ, Horwitz EM. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.
59. Du P, Kibbe WA, Lin SM. nuID: a universal naming scheme of oligonucleotides for Illumina, Affymetrix, and other microarrays. Biology Direct 2007;2.

60. Du P, Kibbe WA, Lin SM. lumi: a pipeline for processing Illumina microarray. Bioinformatics 2008;24:1547-1548.
61. Dunn GP, Bruce AT, Sheehan KC, Shankaran V, Uppaluri R, Bui JD, Diamond MS, Koebel CM, Arthur C, White JM, Schreiber RD. A critical function for type I interferons in cancer immunoediting. Nat Immunol 2005;6:722-729.
62. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nature Reviews Immunology 2006;6:836-848.
63. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002;2:161-174.
64. El-Sherbiny YM, Meade JL, Holmes TD, McGonagle D, Mackie SL, Morgan AW, Cook G, Feyler S, Richards SJ, Davies FE, Morgan GJ, Cook GP. The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. Cancer Res 2007;67:8444-8449.
65. Ewing J. Diffuse endothelioma of bone. Proceedings of the New York Pathological Society 1921;12:17.
66. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759-767.
67. Filen JJ, Filen S, Moulder R, Tuomela S, Ahlfors H, West A, Kouvonen P, Kantola S, Bjorkman M, Katajamaa M, Rasool O, Nyman TA, Lahesmaa R. Quantitative Proteomics Reveals GIMAP Family Proteins 1 and 4 to Be Differentially Regulated during Human T Helper Cell Differentiation. Molecular & Cellular Proteomics 2009;8:32-44.
68. Fiory F, Formisano P, Perruolo G, Beguinot F. Frontiers: PED/PEA-15, a multifunctional protein controlling cell survival and glucose metabolism. Am J Physiol Endocrinol Metab 2009;297:E592-E601.
69. Fischer-Valuck BW, Barrilleaux BL, Phinney DG, Russell KC, Prockop DJ, O'Connor KC. Migratory response of mesenchymal stem cells to macrophage migration inhibitory factor and its antagonist as a function of colony-forming efficiency. Biotechnol Lett 2010;32:19-27.
70. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limon P. The polarization of immune cells in the tumour environment by TGFbeta. Nat Rev Immunol 2010;10:554-567.
71. Fletcher CDM, Unni KK, Mertens F. World Health Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. Lyon: IARC Press, 2002.
72. Forment JV, Kaidi A, Jackson SP. Chromothripsis and cancer: causes and consequences of chromosome shattering. Nat Rev Cancer 2012;12:663-670.
73. Forssell J, Oberg A, Henriksson ML, Stenling R, Jung A, Palmqvist R. High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. Clinical Cancer Research 2007;13:1472-1479.
74. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nature Immunology 2009;10:241-247.
75. Frezzato F, Gattazzo C, Martini V, Trimarco V, Teramo A, Carraro S, Cabrelle A, Ave E, Facco M, Zambello R, Tibaldi E, Brunati AM, Semenzato G, Trentin L. HS1, a Lyn kinase substrate, is abnormally expressed in B-chronic lymphocytic leukemia and correlates with response to fludarabine-based regimen. PLoS ONE 2012;7:e39902.
76. Frings PW, Van Elssen CH, Wieten L, Matos C, Hupperets PS, Schouten HC, Bos GM, van GM. Elimination of the chemotherapy resistant subpopulation of 4T1 mouse breast cancer by haploidentical NK cells cures the vast majority of mice. Breast Cancer Res Treat 2011;130:773-781.
77. Fujiwara T, Bandi M, Nitta M, Ivanova EV, Bronson RT, Pellman D. Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. Nature 2005;437:1043-1047.
78. Fujiwara T, Fukushi J, Yamamoto S, Matsumoto Y, Setsu N, Oda Y, Yamada H, Okada S, Watari K, Ono M, Kuwano M, Kamura S, Iida K, Okada Y, Koga M, Iwamoto Y. Macrophage infiltration predicts a poor prognosis for human ewing sarcoma. Am J Pathol 2011;179:1157-1170.
79. Ganesan LP, Fang HQ, Marsh CB, Tridandapani S. The protein-tyrosine phosphatase SHP-1 associates with the phosphorylated immunoreceptor tyrosine-based activation motif of Fc gamma RIIa to modulate signaling events in myeloid cells. Journal of Biological Chemistry 2003;278:35710-35717.
80. Garcia-Iglesias T, del Toro-Arreola A, barran-Somoza B, del Toro-Arreola S, Sanchez-Hernandez PE, Ramirez-Duenas MG, Balderas-Pena LM, Bravo-Cuellar A, Ortiz-Lazareno PC, neri-Navarro A. Low NKp30, NKp46 and NKG2D expression and reduced cytotoxic activity on NK cells in cervical cancer and precursor lesions. BMC Cancer 2009;9:186.
81. Ge K, Prendergast GC. Bin2, a functionally nonredundant member of the BAR adaptor gene family. Genomics 2000;67:210-220.

82. Geddes K, Magalhaes JG, Girardin SE. Unleashing the therapeutic potential of NOD-like receptors. Nature Reviews Drug Discovery 2009;8:465-479.
83. Gelderblom H, Sydes MR, Morgan RC, Hogendoorn PCW, Lewis IJ, Nooij MA, Taminiau AH, Whelan J. Survival after recurrent osteosarcoma: Data from three European Osteosarcoma Intergroup (EOI) randomized controlled. J Clin Oncol (Meeting Abstracts) 2008;26:10505.
84. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004;5:R80.
85. Gomez TS, McCarney SD, Carrizosa E, Labno CM, Comiskey EO, Nolz JC, Zhu PM, Freedman BD, Clark MR, Rawlings DJ, Billadeau DD, Burkhardt JK. HS1 functions as an essential actin-regulatory adaptor protein at the immune synapse. Immunity 2006;24:741-752.
86. Gordon N, Arndt CAS, Hawkins DS, Dohero DK, Inwards CY, Munsell MF, Stewart J, Koshkina NV, Kleinerman ES. Fas expression in lung metastasis from osteosarcoma patients. J Pediatr Hematol Oncol 2005;27:611-615.
87. Gordon N, Koshkina NV, Jia SF, Khanna C, Mendoza A, Worth LL, Kleinerman ES. Corruption of the Fas pathway delays the pulmonary clearance of murine osteosarcoma cells, enhances their metastatic potential, and reduces the effect of aerosol gemcitabine. Clin Cancer Res 2007;13:4503-4510.
88. Goto M, Miller RW, Ishikawa Y, Sugano H. Excess of rare cancers in Werner syndrome (adult progeria). Cancer Epidemiol Biomarkers Prev 1996;5:239-246.
89. Grimer RJ, Hogendoorn PCW, Vanel D. Tumours of bone: Introduction. In: Fletcher CDM, Bridge JA, Hogendoom PCW, Mertens F, editors. WHO Classification of Tumours of Soft Tissue and Bone. Lyon: IARC, 2013; pp. 244-247.
90. Gulati P, Lemercier C, Guc D, Lappin D, Whaley K. Regulation of the synthesis of C1 subcomponents and C1-inhibitor. Behring Inst Mitt 1993;196-203.
91. Gutwein P, Schramme A, Sinke N, bdel-Bakky MS, Voss B, Obermuller N, Doberstein K, Koziolek M, Fritzsche F, Johannsen M, Jung K, Schaider H, Alteuogt P, Ludwig A, Pfeilschifter J, Kristiansen G. Tumoural CXCL16 expression is a novel prognostic marker of longer survival times in renal cell cancer patients. European Journal of Cancer 2009;45:478-489.
92. Hagemann T, Lawrence T, McNeish I, Charles KA, Kulbe H, Thompson RG, Robinson SC, Balkwill FR. "Re-educating" tumor-associated macrophages by targeting NF-kappa B. Journal of Experimental Medicine 2008;205:1261-1268.
93. Hagemann T, Wilson J, Burke F, Kulbe H, Li NFF, Pluddemann A, Charles K, Gordon S, Balkwill FR. Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype. Journal of Immunology 2006;176:5023-5032.
94. Hallor KH, Staaf J, Bovee JVMG, Hogendoorn PCW, Cleton-Jansen AM, Knuutila S, Savola S, Niini T, Brosjo O, Bauer HCF, von Steyern FV, Jonsson K, Skorpil M, Mandahl N, Mertens F. Genomic Profiling of Chondrosarcoma: Chromosomal Patterns in Central and Peripheral Tumors. Clinical Cancer Research 2009;15:2685-2694.
95. Hansen MF, Koufos A, Gallie BL, Phillips RA, Fodstad O, Brogger A, Gedde-Dahl T, Cavenee WK. Osteosarcoma and retinoblastoma: a shared chromosomal mechanism revealing recessive predisposition. Proc Natl Acad Sci U S A 1985;82:6216-6220.
96. Hansen MF, Seton M, Merchant A. Osteosarcoma in Paget's disease of bone. J Bone Miner Res 2006;21 Suppl 2:58-63.
97. Harting MT, Blakely ML, Jaffe N, Cox CS, Hayes-Jordan A, Benjamin RS, Raymond AK, Andrassy RJ, Lally KP. Long-term survival after aggressive resection of pulmonary metastases among children and adolescents with osteosarcoma. Journal of Pediatric Surgery 2006;41:194-199.
98. Hauben EI, Weeden S, Pringle J, Van Marck EA, Hogendoorn PCW. Does the histological subtype of high-grade central osteosarcoma influence the response to treatment with chemotherapy and does it affect overall survival? A study on 570 patients of two consecutive trials of the European Osteosarcoma Intergroup. European Journal of Cancer 2002;38:1218-1225.
99. He X, Zhu Z, Johnson C, Stoops J, Eaker AE, Bowen W, DeFrances MC. PIK3IP1, a negative regulator of PI3K, suppresses the development of hepatocellular carcinoma. Cancer Research 2008;68:5591-5598.
100. Hervas-Stubbs S, Perez-Gracia JL, Rouzaut A, Sanmamed MF, Le BA, Melero I. Direct effects of type I interferons on cells of the immune system. Clin Cancer Res 2011;17:2619-2627.

101. Hojo S, Koizumi K, Tsuneyama K, Arita Y, Cui Z, Shinohara K, Minami T, Hashimoto I, Nakayama T, Sakurai H, Takano Y, Yoshie O, Tsukada K, Saiki I. High-level expression of chemokine CXCL16 by tumor cells correlates with a good prognosis and increased tumor-infiltrating lymphocytes in colorectal cancer. Cancer Research 2007;67:4725-4731.
102. Holland AJ, Cleveland DW. Chromoanagenesis and cancer: mechanisms and consequences of localized, complex chromosomal rearrangements. Nat Med 2012;18:1630-1638.
103. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol 2006;6:644-658.
104. Honorati MC, Neri S, Cattini L, Facchini A. IL-17 enhances the susceptibility of U-2OS osteosarcoma cells to NK cell lysis. Clin Exp Immunol 2003;133:344-349.
105. Horvai A, Unni KK. Premalignant conditions of bone. Journal of Orthopaedic Science 2006;11:412-423.
106. Inui M, Kikuchi Y, Aoki N, Endo S, Maeda T, Sugahara-Tobinai A, Fujimura S, Nakamura A, Kumanogoh A, Colonna M, Takai T. Signal adaptor DAP10 associates with MDL-1 and triggers osteoclastogenesis in cooperation with DAP12. Proceedings of the National Academy of Sciences of the United States of America 2009;106:4816-4821.
107. Izadpanah R, Kaushal D, Kriedt C, Tsien F, Patel B, Dufour J, Bunnell BA. Long-term in vitro expansion alters the biology of adult mesenchymal stem cells. Cancer Res 2008;68:4229-4238.
108. Jacquet S, Malaval C, Martinez LO, Sak K, Rolland C, Perez C, Nauze M, Champagne E, Terce F, Gachet C, Perret B, Collet X, Boeynaems JM, Barbaras R. The nucleotide receptor P2Y(13) is a key regulator of hepatic high-density lipoprotein (HDL) endocytosis. Cellular and Molecular Life Sciences 2005;62:2508-2515.
109. Jaiswal S, Chao MP, Majeti R, Weissman IL. Macrophages as mediators of tumor immunosurveillance. Trends Immunol 2010;31:212-219.
110. Jan M, Majeti R. Clonal evolution of acute leukemia genomes. Oncogene 2013;32:135-140.
111. Jarvinen A, Keinanen TA, Grigorenko NA, Khomutov AR, Uimari A, Vepsalainen J, Narvanen A, Alhonen L, Janne J. Guide molecule-driven stereospecific degradation of alpha-methylpolyamines by polyamine oxidase. Journal of Biological Chemistry 2006;281:4589-4595.
112. Jensen TO, Schmidt H, Moller HJ, Hoyer M, Maniecki MB, Sjoegren P, Christensen IJ, Steiniche T. Macrophage Markers in Serum and Tumor Have Prognostic Impact in American Joint Committee on Cancer Stage I/II Melanoma. Journal of Clinical Oncology 2009;27:3330-3337.
113. Jeong JO, Han JW, Kim JM, Cho HJ, Park C, Lee N, Kim DW, Yoon YS. Malignant tumor formation after transplantation of short-term cultured bone marrow mesenchymal stem cells in experimental myocardial infarction and diabetic neuropathy. Circ Res 2011;108:1340-1347.
114. Jeys LM, Grimer RJ, Carter SR, Tillman RM, Abudu A. Post operative infection and increased survival in osteosarcoma patients: Are they associated? Annals of Surgical Oncology 2007;14:2887-2895.
115. Johannessen M, Moller S, Hansen T, Moens U, Van Ghelue M. The multifunctional roles of the four-and-a-half-LIM only protein FHL2. Cellular and Molecular Life Sciences 2006;63:268-284.
116. Josse C, Schoemans R, Niessen NA, Delgaudine M, Hellin AC, Herens C, Delvenne P, Bours V. Systematic chromosomal aberrations found in murine bone marrow-derived mesenchymal stem cells. Stem Cells Dev 2010;19:1167-1173.
117. Kansara M, Thomas DM. Molecular pathogenesis of osteosarcoma. DNA Cell Biol 2007;26:1-18.
118. Karre K. Natural killer cell recognition of missing self. Nat Immunol 2008;9:477-480.
119. Kauffmann A, Gentleman R, Huber W. arrayQualityMetrics--a bioconductor package for quality assessment of microarray data. Bioinformatics 2009;25:415-416.
120. Kayton ML, Huvos AG, Casher J, Abramson SJ, Rosen NS, Wexler LH, Meyers P, LaQuaglia MP. Computed tomographic scan of the chest underestimates the number of metastatic lesions in osteosarcoma. Journal of Pediatric Surgery 2006;41:200-204.
121. Kempf-Bielack B, Bielack SS, Jurgens H, Branscheid D, Berdel WE, Exner GU, Gobel U, Helmke K, Jundt G, Kabisch H, Kevric M, Klingebiel T, Kotz R, Maas R, Schwarz R, Semik M, Treuner J, Zoubek A, Winkler K. Osteosarcoma relapse after combined modality therapy: An analysis of unselected patients in the Cooperative Osteosarcoma Study Group (COSS). Journal of Clinical Oncology 2005;23:559-568.
122. Kim DW, Min HS, Lee KH, Kim YJ, Oh DY, Jeon YK, Lee SH, Im SA, Chung DH, Kim YT, Kim TY, Bang YJ, Sung SW, Kim JH, Heo DS. High tumour islet macrophage infiltration correlates with improved patient survival but not

with EGFR mutations, gene copy number or protein expression in resected non-small cell lung cancer. British Journal of Cancer 2008;98:1118-1124.

123. Kim J, Kang JW, Park JH, Choi Y, Choi KS, Park KD, Baek DH, Seong SK, Min HK, Kim HS. Biological characterization of long-term cultured human mesenchymal stem cells. Arch Pharm Res 2009;32:117-126.
124. Kim JH, Chu FC, Woodward HQ, Huvos A. Radiation induced sarcomas of bone following therapeutic radiation. Int J Radiat Oncol Biol Phys 1983;9:107-110.
125. Klein MJ, Parisien MV, Schneider-Stock R. Osteogenic Tumours. In: Fletcher CDM, Unni KK, Mertens F, editors. World Health Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. Lyon: IARC Press, 2002; pp. 259-285.
126. Kleinerman ES, Raymond AK, Bucana CD, Jaffe N, Harris MB, Krakoff IH, Benjamin R, Fidler IJ. Unique Histological-Changes in Lung Metastases of Osteosarcoma Patients Following Therapy with Liposomal Muramyl Tripeptide (Cgp-19835A Lipid). Cancer Immunology Immunotherapy 1992;34:211-220.
127. Kolk SM, Pasterkamp RJ. MICAL flavoprotein monooxygenases: Structure, function and role in semaphorin signaling. Semiaphorins: Receptor and Intracellular Signaling Mechanisms 2007;600:38-51.
128. Konjevic G, Mirjacic MK, Vuletic A, Jovic V, Jurisic V, Babovic N, Spuzic I. Low expression of CD161 and NKG2D activating NK receptor is associated with impaired NK cell cytotoxicity in metastatic melanoma patients. Clin Exp Metastasis 2007;24:1-11.
129. Kono H, Rock KL. How dying cells alert the immune system to danger. Nature Reviews Immunology 2008;8:279-289.
130. Koshkina NV, Khanna C, Mendoza A, Guan H, DeLauter L, Kleinerman ES. Fas-negative osteosarcoma tumor cells are selected during metastasis to the lungs: The role of the fas pathway in the metastatic process of osteosarcoma. Mol Cancer Res 2007;5:991-999.
131. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. Nat Rev Cancer 2012;12:860-875.
132. Kubista B, Trieb K, Blahovec H, Kotz R, Micksche M. Hyperthermia increases the susceptibility of chondro- and osteosarcoma cells to natural killer cell-mediated lysis. Anticancer Res 2002;22:789-792.
133. Kubo T, Shimose S, Matsuo T, Fujimori J, Arihiro K, Ochi M. Interferon-alpha/beta receptor as a prognostic marker in osteosarcoma. J Bone Joint Surg Am 2011;93:519-526.
134. Kurzman ID, MacEwen EG, Rosenthal RC, Fox LE, Keller ET, Helfand SC, Vail DM, Dubielzig RR, Madewell BR, Rodriguez CO, Obradovich J, Fidel J, Rosenberg M. Adjuvant therapy for osteosarcoma in dogs: Results of randomized clinical trials using combined liposome-encapsulated muramyl tripeptide and cisplatin. Clinical Cancer Research 1995;1:1595-1601.
135. Lakshmikanth T, Burke S, Ali TH, Kimpfler S, Ursini F, Ruggeri L, Capanni M, Umansky V, Paschen A, Sucker A, Pende D, Groh V, Biassoni R, Hoglund P, Kato M, Shibuya K, Schadendorf D, Anichini A, Ferrone S, Velardi A, Karre K, Shibuya A, Carbone E, Colucci F. NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo. J Clin Invest 2009;119:1251-1263.
136. Lascelles BD, Dernell WS, Correa MT, Lafferty M, Devitt CM, Kuntz CA, Straw RC, Withrow SJ. Improved survival associated with postoperative wound infection in dogs treated with limb-salvage surgery for osteosarcoma. Ann Surg Oncol 2005;12:1073-1083.
137. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579-1586.
138. Lee CH, Espinosa I, Vrijaldenhoven S, Subramanian S, Montgomery KD, Zhu S, Marinelli RJ, Peterse JL, Poulin N, Nielsen TO, West RB, Gilks CB, van de Rijn M. Prognostic significance of macrophage infiltration in leiomyosarcomas. Clinical Cancer Research 2008;14:1423-1430.
139. Lesinski GB, Badgwell B, Zimmerer J, Crespin T, Hu Y, Abood G, Carson WE, III. IL-12 pretreatments enhance IFN-alpha-induced Janus kinase-STAT signaling and potentiate the antitumor effects of IFN-alpha in a murine model of malignant melanoma. J Immunol 2004;172:7368-7376.
140. Lesinski GB, Kondadasula SV, Crespin T, Shen L, Kendra K, Walker M, Carson WE, III. Multiparametric flow cytometric analysis of inter-patient variation in STAT1 phosphorylation following interferon Alfa immunotherapy. J Natl Cancer Inst 2004;96:1331-1342.

141. Lewis IJ, Nooij MA, Whelan J, Sydes MR, Grimer R, Hogendoorn PCW, Memon MA, Weeden S, Uscinska BM, van Glabbeke M, Kirkpatrick A, Hauben EI, Craft AW, Taminiau AHM. Improvement in histologic response but not survival in osteosarcoma patients treated with intensified chemotherapy: A randomized phase III trial of the European Osteosarcoma Intergroup. J Natl Cancer Inst 2007;99:112-128.

142. Lewis JS, Landers RJ, Underwood JC, Harris AL, Lewis CE. Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas. J Pathol 2000;192:150-158.

143. Liebau C, Merk H, Schmidt S, Roesel C, Karreman C, Prisack JB, Bojar H, Baltzer AWA. Interleukin-12 and interleukin-18 change ICAM-1 expression, and enhance natural killer cell mediated cytolysis of human osteosarcoma cells. Cytokines Cell Mol Ther 2002;7:135-142.

144. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, Qian H, Xue XN, Pollard JW. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Research 2006;66:11238-11246.

145. Lin SM, Du P, Huber W, Kibbe WA. Model-based variance-stabilizing transformation for Illumina microarray data. Nucleic Acids Research 2008;36.

146. Lissbrant IF, Stattin P, Wikstrom P, Damber JE, Egevad L, Bergh A. Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival. Int J Oncol 2000;17:445-451.

147. Liu H, Shalev I, Manuel J, He W, Leung E, Crookshank J, Liu MF, Diao J, Cattral M, Clark DA, Isenman DE, Gorczynski RM, Grant DR, Zhang L, Phillips MJ, Cybulsky MI, Levy GA. The FGL2-FcgammaRIIB pathway: a novel mechanism leading to immunosuppression. Eur J Immunol 2008;38:3114-3126.

148. Ljunggren HG, Malmberg KJ. Prospects for the use of NK cells in immunotherapy of human cancer. Nat Rev Immunol 2007;7:329-339.

149. Loges NT, Olbrich H, Fenske L, Mussaffi H, Horvath J, Fliegauf M, Kuhl H, Baktai G, Peterffy E, Chodhari R, Chung EMK, Rutman A, O'Callaghan C, Blau H, Tiszlavicz L, Voelkel K, Witt M, Zietkiewicz E, Neesen J, Reinhardt R, Mitchison HM, Omran H. DNAI2 Mutations Cause Primary Ciliary Dyskinesia with Defects in the Outer Dynein Arm. American Journal of Human Genetics 2008;83:547-558.

150. Loiselle FB, Morgan PE, Alvarez BV, Casey JR. Regulation of the human NBC3 Na+/HCO3- cotransporter by carbonic anhydrase II and PKA. American Journal of Physiology-Cell Physiology 2004;286:C1423-C1433.

151. Luksch R, Perotti D, Cefalo G, Passerini CG, Massimino M, Spreafico F, Casanova M, Ferrari A, Terenziani M, Polastri D, Gambirasio F, Podda M, Bozzi F, Ravagnani F, Parmiani G, Bellani FF. Immunomodulation in a treatment program including pre- and post-operative interleukin-2 and chemotherapy for childhood osteosarcoma. Tumori 2003;89:263-268.

152. Ma CS, Nichols KE, Tangye SG. Regulation of cellular and humoral immune responses by the SLAM and SAP families of molecules. Annual Review of Immunology 2007;25:337-379.

153. Man TK, Chintagumpala M, Visvanathan J, Shen J, Perlaky L, Hicks J, Johnson M, Davino N, Murray J, Helman L, Meyer W, Triche T, Wong KK, Lau CC. Expression profiles of osteosarcoma that can predict response to chemotherapy. Cancer Res 2005;65:8142-8150.

154. Manara MC, Serra M, Benini S, Picci P, Scotlandi K. Effectiveness of Type I interferons in the treatment of multidrug resistant osteosarcoma cells. Int J Oncol 2004;24:365-372.

155. Maran A, Shogren KL, Benedikt M, Sarkar G, Turner RT, Yaszemski MJ. 2-methoxyestradiol-induced cell death in osteosarcoma cells is preceded by cell cycle arrest (vol 104, pg 1937, 2008). Journal of Cellular Biochemistry 2008;105:1146.

156. Mariani E, Meneghetti A, Tarozzi A, Cattini L, Facchini A. Interleukin-12 induces efficient lysis of natural killer-sensitive and natural killer-resistant human osteosarcoma cells: The synergistic effect of interleukin-2. Scand J Immunol 2000;51:618-625.

157. Mariani E, Tarozzi A, Meneghetti A, Cattini L, Facchini A. Human osteosarcoma cell susceptibility to natural killer cell lysis depends on CD54 and increases after TNF alpha incubation. FEBS Lett 1997;406:83-88.

158. Mariani E, Tarozzi A, Meneghetti A, Cattini L, Facchini A. TNF-alpha but not IL-1 and IL-6 modifies the susceptibility of human osteosarcoma cells to NK lysis. Int J Oncol 1998;13:349-353.

159. Mariani E, Tarozzi A, Meneghetti A, Tadolini M, Facchini A. Lytic activity of IL-2 and IL-12 stimulated NK cells against HOS osteosarcoma cell line. Boll Soc Ital Biol Sper 1996;72:21-27.

160. Marina N, Bielack S, Whelan J, Smeland S, Krailo M, Sydes MR, Butterfass-Bahloul T, Calaminus G, Bernstein M. International collaboration is feasible in trials for rare conditions: the EURAMOS experience. Cancer Treat Res 2009;152:339-353.

161. Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: New molecules and patterns of gene expression. Journal of Immunology 2006;177:7303-7311.
162. Matsumura S, Wang BM, Kawashima N, Braunstein S, Badura M, Cameron TO, Babb JS, Schneider RJ, Formenti SC, Dustin ML, Demaria S. Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. Journal of Immunology 2008;181:3099-3107.
163. McDermott DF. Immunotherapy of metastatic renal cell carcinoma. Cancer 2009;115:2298-2305.
164. McHale CM, Smith MT. Prenatal origin of chromosomal translocations in acute childhood leukemia: implications and future directions. Am J Hematol 2004;75:254-257.
165. Meneghetti A, Mariani E, Santi S, Riccio M, Cattini L, Paoletti S, Facchini A. NK binding capacity and lytic activity depend on the expression of ICAM-1 on target bone tumours. Int J Oncol 1999;15:909-914.
166. Meng W, Mushika Y, Ichii T, Takeichi M. Anchorage of Microtubule Minus Ends to Adherens Junctions Regulates Epithelial Cell-Cell Contacts. Cell 2008;135:948-959.
167. Meng X, Fan J, Shen Z. Roles of BCCIP in chromosome stability and cytokinesis. Oncogene 2007;26:6253-6260.
168. Mey U, Strehl J, Gorschluter M, Ziske C, Glasmacher A, Pralle H, Schmidt-Wolf I. Advances in the treatment of hairy-cell leukaemia. Lancet Oncol 2003;4:86-94.
169. Meyers PA, Schwartz CL, Krailo M, Kleinerman ES, Betcher D, Bernstein ML, Conrad E, Ferguson W, Gebhardt M, Goorin AM, Harris MB, Healey J, Huvos A, Link M, Montebello J, Nadel H, Nieder M, Sato J, Siegal G, Weiner M, Wells R, Wold L, Womer R, Grier H. Osteosarcoma: A randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate. J Clin Oncol 2005;23:2004-2011.
170. Meyers PA, Schwartz CL, Krailo MD, Healey JH, Bernstein ML, Betcher D, Ferguson WS, Gebhardt MC, Goorin AM, Harris M, Kleinerman E, Link MP, Nadel H, Nieder M, Siegal GP, Weiner MA, Wells RJ, Womer RB, Grier HE. Osteosarcoma: The addition of muramyl tripeptide to chemotherapy improves overall survival - A report from the Children's Oncology Group. J Clin Oncol 2008;26:633-638.
171. Migeotte I, Communi D, Parmentier M. Formyl peptide receptors: A promiscuous subfamily of G protein-coupled receptors controlling immune responses. Cytokine & Growth Factor Reviews 2006;17:501-519.
172. Miller BJ, Cram P, Lynch CF, Buckwalter JA. Risk Factors for Metastatic Disease at Presentation with Osteosarcoma: An Analysis of the SEER Database. J Bone Joint Surg Am 2013;95:e891-e898.
173. Miller JS, Cooley S, Parham P, Farag SS, Verneris MR, McQueen KL, Guethlein LA, Trachtenberg EA, Haagenson M, Horowitz MM, Klein JP, Weisdorf DJ. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. Blood 2007;109:5058-5061.
174. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, McKenna D, Le C, Defor TE, Burns LJ, Orchard PJ, Blazar BR, Wagner JE, Slungaard A, Weisdorf DJ, Okazaki IJ, McGlave PB. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood 2005;105:3051-3057.
175. Miller TT. Bone tumors and tumorlike conditions: Analysis with conventional radiography. Radiology 2008;246:662-674.
176. Mintz MB, Sowers R, Brown KM, Hilmer SC, Mazza B, Huvos AG, Meyers PA, LaFleur B, McDonough WS, Henry MM, Ramsey KE, Antonescu CR, Chen W, Healey JH, Daluski A, Berens ME, MacDonald TJ, Gorlick R, Stephan DA. An expression signature classifies chemotherapy-resistant pediatric osteosarcoma. Cancer Research 2005;65:1748-1754.
177. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer 2009;115:1531-1543.
178. Mistry AR, O'Callaghan CA. Regulation of ligands for the activating receptor NKG2D. Immunology 2007;121:439-447.
179. Miura M, Miura Y, Padilla-Nash HM, Molinolo AA, Fu BJ, Patel V, Seo BM, Sonoyama W, Zheng JJ, Baker CC, Chen WJ, Ried T, Shi ST. Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells 2006;24:1095-1103.
180. Miura Y, Shimazu R, Miyake K, Akashi S, Ogata H, Yamashita Y, Narisawa Y, Kimoto M. RP105 is associated with MD-1 and transmits an activation signal in human B cells. Blood 1998;92:2815-2822.
181. Mohseny AB, Hogendoorn PCW. Concise review: mesenchymal tumors: when stem cells go mad. Stem Cells 2011;29:397-403.

182. Mohseny AB, Hogendoorn PCW, Cleton-Jansen AM. Osteosarcoma models: from cell lines to zebrafish. Sarcoma 2012;2012:417271.

183. Mohseny AB, Szuhai K, Romeo S, Buddingh EP, Briaire-de Bruijn I, de Jong D, van Pel M, Cleton-Jansen AM, Hogendoorn PCW. Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. J Pathol 2009;219:294-305.

184. Moldenhauer G, Henne C, Karhausen J, Moller P. Surface-expressed invariant chain (CD74) is required for internalization of human leucocyte antigen-DR molecules to early endosomal compartments. Immunology 1999;96:473-484.

185. Moog-Lutz C, Cave-Riant F, Guibal FC, Breau MA, Di Gioia Y, Couraud PO, Cayre YE, Bourdoulous S, Lutz PG. JAML, a novel protein with characteristics of a junctional adhesion molecule, is induced during differentiation of myeloid leukemia cells. Blood 2003;102:3371-3378.

186. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, Biassoni R, Moretta L. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 2001;19:197-223.

187. Moretti S, Procopio A, Boemi M, Catalano A. Neuronal semaphorins regulate a primary immune response. Current Neurovascular Research 2006;3:295-305.

188. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008;8:958-969.

189. Mulcahy H, O'Rourke KP, Adams C, Molloy MG, O'Gara F. LST1 and NCR3 expression in autoimmune inflammation and in response to IFN-gamma, LPS and microbial infection. Immunogenetics 2006;57:893-903.

190. Muller CR, Smeland S, Bauer HC, Saeter G, Strander H. Interferon-alpha as the only adjuvant treatment in high-grade osteosarcoma: long term results of the Karolinska Hospital series. Acta Oncol 2005;44:475-480.

191. Nakashima Y, Deie M, Yanada S, Sharman P, Ochi M. Magnetically labeled human natural killer cells, accumulated in vitro by an external magnetic force, are effective against HOS osteosarcoma cells. Int J Oncol 2005;27:965-971.

192. Nakayama M, Akiba H, Takeda K, Kojima Y, Hashiguchi M, Azuma M, Yagita H, Okumura K. Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. Blood 2009;113:3821-3830.

193. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Metivier D, Larochette N, van Endert P, Ciccosanti F, Piacentini M, Zitvogel L, Kroemer G. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nature Medicine 2007;13:54-61.

194. Ochi K, Daigo Y, Katagiri T, Nagayama S, Tsunoda T, Myoui A, Naka N, Araki N, Kudawara I, Ieguchi M, Toyama Y, Toguchida J, Yoshikawa H, Nakamura Y. Prediction of response to neoadjuvant chemotherapy for osteosarcoma by gene-expression profiles. Int J Oncol 2004;24:647-655.

195. Ojalvo LS, King W, Cox D, Pollard JW. High-Density Gene Expression Analysis of Tumor-Associated Macrophages from Mouse Mammary Tumors. American Journal of Pathology 2009;174:1048-1064.

196. Okazaki H, Igarashi M, Nishi M, Sekiya M, Tajima M, Takase S, Takanashi M, Ohta K, Tamura Y, Okazaki S, Yahagi N, Ohashi K, memiya-Kudo M, Nakagawa Y, Nagai R, Kadowaki T, Osuga J, Ishibashi S. Identification of Neutral Cholesterol Ester Hydrolase, a Key Enzyme Removing Cholesterol from Macrophages. Journal of Biological Chemistry 2008;283:33357-33364.

197. Olety B, Walte M, Honnert U, Schillers H, Bahler M. Myosin 1G (Myo1G) is a haematopoietic specific myosin that localises to the plasma membrane and regulates cell elasticity. FEBS Lett 2009.

198. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701-705.

199. Ottaviano L, Schaefer KL, Gajewski M, Huckenbeck W, Baldus S, Rogel U, Mackintosh C, de Alava E, Myklebost O, Kresse SH, Meza-Zepeda LA, Serra M, Cleton-Jansen AM, Hogendoorn PCW, Buerger H, Aigner T, Gabbert HE, Poremba C. Molecular Characterization of Commonly Used Cell Lines for Bone Tumor Research: A Trans-European EuroBoNet Effort. Genes Chromosomes Cancer 2010;49:40-51.

200. Pahl JH, Ruslan SE, Buddingh EP, Santos SJ, Szuhai K, Serra M, Gelderblom H, Hogendoorn PCW, Egeler RM, Schilham MW, Lankester AC. Anti-EGFR antibody cetuximab enhances the cytolytic activity of natural killer cells toward osteosarcoma. Clin Cancer Res 2012;18:432-441.

201. Pampalona J, Frias C, Genesca A, Tusell L. Progressive telomere dysfunction causes cytokinesis failure and leads to the accumulation of polyploid cells. PLoS Genet 2012;8:e1002679.

202. Pansuriya TC, van ER, d'Adamo P, van Ruler MA, Kuijjer ML, Oosting J, Cleton-Jansen AM, van Oosterwijk JG, Verbeke SL, Meijer D, van WT, Nord KH, Sangiorgi L, Toker B, Liegl-Atzwanger B, San-Julian M, Sciot R,

Limaye N, Kindblom LG, Daugaard S, Godfraind C, Boon LM, Vikkula M, Kurek KC, Szuhai K, French PJ, Bovee JV. Somatic mosaic IDH1 and IDH2 mutations are associated with enchondroma and spindle cell hemangioma in Ollier disease and Maffucci syndrome. Nat Genet 2011;43:1256-1261.

203. Parkhurst MR, Riley JP, Dudley ME, Rosenberg SA. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. Clin Cancer Res 2011;17:6287-6297.
204. Pasello M, Michelacci F, Scionti I, Hattinger CM, Zuntini M, Caccuri AM, Scotlandi K, Picci P, Serra M. Overcoming glutathione S-transferase P1-related cisplatin resistance in osteosarcoma. Cancer Res 2008;68:6661-6668.
205. Petrilli AS, Gentil FC, Epelman S, Lopes LF, Bianchi A, Lopes A, Figueiredo MTD, Marques E, Debellis N, Consentino E, Prospero D, Decamargo OP, Oliveira NR, Franco E, Jaffe N. Increased Survival, Limb Preservation, and Prognostic Factors for Osteosarcoma. Cancer 1991;68:733-737.
206. Picci P, Sieberova G, Alberghini M, Balladelli A, Vanel D, Hogendoorn PCW, Mercuri M. Late sarcoma development after curettage and bone grafting of benign bone tumors. Eur J Radiol 2011;77:19-25.
207. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 2005;5:375-386.
208. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nature Reviews Cancer 2004;4:71-78.
209. Prockop DJ, Keating A. Relearning the lessons of genomic stability of human cells during expansion in culture: implications for clinical research. Stem Cells 2012;30:1051-1052.
210. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell 2010;141:39-51.
211. R Foundation for Statistical Computing. R: A language and environment for statistical computing, reference index version 2.9.0. 2005. Vienna, Austria. (Computer Program).
212. Rashid S, Pilecka I, Torun A, Olchowik M, Bielinska B, Miaczynska M. Endosomal Adaptor Proteins APPL1 and APPL2 Are Novel Activators of beta-Catenin/TCF-mediated Transcription. Journal of Biological Chemistry 2009;284:18115-18128.
213. Raulet DH, Vance RE. Self-tolerance of natural killer cells. Nature Reviews Immunology 2006;6:520-531.
214. Raymond AK, Ayala AG, Knuutila S. Conventional osteosarcoma. In: Fletcher CDM, Unni KK, editors. World Health Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. Lyon: IARC Press, 2002; pp. 264-270.
215. Riihonen R, Song I, Nielsen S, Laitala-Leinonen T, Kwon T. Electroneutral sodium/bicarbonate cotransporter NBCn1 is expressed in rat osteoclast ruffled border membrane. Journal of Bone and Mineral Research 2007;22:S253.
216. Rissoan MC, Duhen T, Bridon JM, driss-Vermare N, Peronne C, de Saint Vis B, Briere F, Bates EEM. Subtractive hybridization reveals the expression of immunoglobulinlike transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells. Blood 2002;100:3295-3303.
217. Rosenberg AE, Cleton-Jansen AM, de Pinieux G, Deyrup AT, Hauben E, Squire J. Conventional osteosarcoma. In: Fletcher CDM, Bridge JA, Hogendoom PCW, Mertens F, editors. WHO Classification of Tumours of Soft Tissue and Bone. Lyon: IARC, 2013; pp. 282-288.
218. Rosland GV, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwietz J, Tonn JC, Goldbrunner R, Lonning PE, Bjerkvig R, Schichor C. Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. Cancer Res 2009;69:5331-5339.
219. Ross AL, Leder DE, Weiss J, Izakovic J, Grichnik JM. Genomic instability in cultured stem cells: associated risks and underlying mechanisms. Regen Med 2011;6:653-662.
220. Rozeman LB, Hameetman L, Cleton-Jansen AM, Taminiau AHM, Hogendoorn PCW, Bovee JVMG. Absence of IHH and retention of PTHrP signalling in enchondromas and central chondrosarcomas. Journal of Pathology 2005;205:476-482.
221. Rubio D, Garcia S, De la Cueva T, Paz MF, Lloyd AC, Bernad A, Garcia-Castro J. Human mesenchymal stem cell transformation is associated with a mesenchymal-epithelial transition. Experimental Cell Research 2008;314:691-698.
222. Rubio D, Garcia S, Paz MF, De la CT, Lopez-Fernandez LA, Lloyd AC, Garcia-Castro J, Bernad A. Molecular characterization of spontaneous mesenchymal stem cell transformation. PLoS ONE 2008;3:e1398.
223. Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC, Bernad A. Spontaneous human adult stem cell transformation. Cancer Research 2005;65:3035-3039.

224. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F, Martelli MF, Velardi A. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002;295:2097-2100.

225. Ruiter DJ, Ferrier CM, van Muijen GNP, Henzen-Logmans SC, Kennedy S, Kramer MD, Nielsen BS, Schmitt M. Quality control of immunohistochemical evaluation of tumour-associated plasminogen activators and related components. Eur J Cancer 1998;34:1334-1340.

226. Saarikoski ST, Rivera SP, Hankinson O, Husgafvel-Pursiainen K. CYP2S1: a short review. Toxicol Appl Pharmacol 2005;207:62-69.

227. Saeter G, Elomaa I, Wahlqvist Y, Alvegard TA, Wiebe T, Monge O, Forrestier E, Solheim OP. Prognostic factors in bone sarcomas. Acta Orthopaedica Scandinavica 1997;68:156-160.

228. Saeter G, Hoie J, Stenwig AE, Johansson AK, Hannisdal E, Solheim OP. Systemic Relapse of Patients with Osteogenic-Sarcoma. Cancer 1995;75:1084-1093.

229. Salas S, Jezequel P, Campion L, Deville JL, Chibon F, Bartoli C, Gentet JC, Charbonnel C, Gouraud W, Voutsinos-Porche B, Brouchet A, Duffaud F, Figarella-Branger D, Bouvier C. Molecular characterization of the response to chemotherapy in conventional osteosarcomas: predictive value of HSD17B10 and IFITM2. Int J Cancer 2009;125:851-860.

230. Sandberg AA, Bridge JA. Updates on cytogenetics and molecular genetics of bone and soft tissue tumors: Ewing sarcoma and peripheral primitive neuroectodermal tumors. Cancer Genet Cytogenet 2000;123:1-26.

231. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. Cancer Genetics and Cytogenetics 2003;145:1-30.

232. Schwartz V, Lue HQ, Kraemer S, Korbiel J, Krohn R, Ohl K, Bucala R, Weber C, Bernhagen J. A functional heteromeric MIF receptor formed by CD74 and CXCR4. Febs Letters 2009;583:2749-2757.

233. Scielzo C, Ghia P, Conti A, Bachi A, Guida G, Geuna M, Alessio M, Caligaris-Cappio F. HS1 protein is differentially expressed in chronic lymphocytic leukemia patient subsets with good or poor prognoses. J Clin Invest 2005;115:1644-1650.

234. Screpanti V, Wallin RPA, Ljunggren HG, Grandien A. A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. J Immunol 2001;167:2068-2073.

235. Serra M, Reverter-Branch, Maurici D, Benini S, Shen JN, Chano T, Hattinger CM, Manara MC, Pasello M, Scotlandi K, Picci P. Analysis of dihydrofolate reductase and reduced folate carrier gene status in relation to methotrexate resistance in osteosarcoma cells. Ann Oncol 2004;15:151-160.

236. Serra M, Scotlandi K, Manara MC, Maurici D, Lollini PL, Degiovanni C, Toffoli G, Baldini N. Establishment and Characterization of Multidrug-Resistant Human Osteosarcoma Cell-Lines. Anticancer Res 1993;13:323-329.

237. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, Biswas SK, Allavena P, Mantovani A. Macrophage polarization in tumour progression. Seminars in Cancer Biology 2008;18:349-355.

238. Sica A, Saccani A, Bottazzi B, Polentarutti N, Vecchi A, van DJ, Mantovani A. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. J Immunol 2000;164:762-767.

239. Sinha P, Clements VK, Ostrand-Rosenberg S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. Journal of Immunology 2005;174:636-645.

240. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:Article3.

241. Sorensen AL, Rumjantseva V, Nayeb-Hashemi S, Clausen H, Hartwig JH, Wandall HH, Hoffmeister KM. Role of sialic acid for platelet life span: exposure of beta-galactose results in the rapid clearance of platelets from the circulation by asialoglycoprotein receptor-expressing liver macrophages and hepatocytes. Blood 2009;114:1645-1654.

242. Sottnik JL, U'ren LW, Thamm DH, Withrow SJ, Dow SW. Chronic bacterial osteomyelitis suppression of tumor growth requires innate immune responses. Cancer Immunol Immunother 2010;59:367-378.

243. Souhami RL, Craft AW, VanderEijken JW, Nooij M, Spooner D, Bramwell VHC, Wierzbicki R, Malcolm AJ, Kirkpatrick A, Uscinska BM, VanGlabbeke M, Machin D. Randomised trial of two regimens of chemotherapy in operable osteosarcoma: a study of the European Osteosarcoma Intergroup. Lancet 1997;350:911-917.

244. Spierings E, Wieles B, Goulmy E. Minor histocompatibility antigens big in tumour therapy. Trends in Immunology 2004;25:56-60.

245. Spilsbury K, Omara MA, Wu WM, Rowe PB, Symonds G, Takayama Y. Isolation of A Novel Macrophage-Specific Gene by Differential Cdna Analysis. Blood 1995;85:1620-1629.
246. Starnes CO. Coley's toxins in perspective. Nature 1992;357:11-12.
247. Stephens PJ, Greenman CD, Fu B, Yang F, Bignell GR, Mudie LJ, Pleasance ED, Lau KW, Beare D, Stebbings LA, McLaren S, Lin ML, McBride DJ, Varela I, Nik-Zainal S, Leroy C, Jia M, Menzies A, Butler AP, Teague JW, Quail MA, Burton J, Swerdlow H, Carter NP, Morsberger LA, Iacobuzio-Donahue C, Follows GA, Green AR, Flanagan AM, Stratton MR, Futreal PA, Campbell PJ. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell 2011;144:27-40.
248. Stiller CA, Bielack SS, Jundt G, Steliarova-Foucher E. Bone tumours in European children and adolescents, 1978-1997. Report from the Automated Childhood Cancer Information System Project. European Journal of Cancer 2006;42:2124-2135.
249. Strander H. Interferons and osteosarcoma. Cytokine & Growth Factor Reviews 2007;18:373-380.
250. Su KH, Yang HX, Li XR, Li XL, Gibson AW, Cafardi JM, Zhou T, Edberg JC, Kimberly RP. Expression profile of Fc gamma RIIB on leusocytes and its dysregulation in systemic lupus erythematosus. Journal of Immunology 2007;178:3272-3280.
251. Szuhai K, Cleton-Jansen AM, Hogendoorn PCW, Bovee JV. Molecular pathology and its diagnostic use in bone tumors. Cancer Genet 2012;205:193-204.
252. Szuhai K, Ijszenga M, Tanke HJ, Rosenberg C, Hogendoorn PCW. Molecular cytogenetic characterization of four previously established and two newly established Ewing sarcoma cell lines. Cancer Genet Cytogenet 2006;166:173-179.
253. Szuhai K, Tanke HJ. COBRA: combined binary ratio labeling of nucleicacid probes for multi-color fluorescence in situ hybridization karyotyping. Nature Protocols 2006;1:264-275.
254. Tarte K, Gaillard J, Lataillade JJ, Fouillard L, Becker M, Mossafa H, Tchirkov A, Rouard H, Henry C, Splingard M, Dulong J, Monnier D, Gourmelon P, Gorin NC, Sensebe L. Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. Blood 2010;115:1549-1553.
255. Terme M, Ullrich E, Delahaye NF, Chaput N, Zitvogel L. Natural killer cell-directed therapies: moving from unexpected results to successful strategies. Nat Immunol 2008;9:486-494.
256. Thompson RC, Cheng EY, Clohisy DR, Perentesis J, Manivel C, Le CT. Results of treatment for metastatic osteosarcoma with neoadjuvant chemotherapy and surgery. Clinical Orthopaedics and Related Research 2002;240-247.
257. Tian Y, Jain S, Kelemen SE, Autieri MV. AIF-1 expression regulates endothelial cell activation, signal transduction, and vasculogenesis. American Journal of Physiology-Cell Physiology 2009;296:C256-C266.
258. Tian Y, Kelemen SE, Autieri MV. Inhibition of AIF-1 expression by constitutive siRNA expression reduces macrophage migration, proliferation, and signal transduction initiated by atherogenic stimuli. American Journal of Physiology-Cell Physiology 2006;290:C1083-C1091.
259. Tolar J, Nauta AJ, Osborn MJ, Mortari AP, McElmurry RT, Bell S, Xia LL, Zhou N, Riddle M, Schroeder TM, Westendorf JJ, Mcivor RS, Hogendoorn PCW, Szuhai K, Oseth L, Hirsch B, Yant SR, Kay MA, Peister A, Prockop DJ, Fibbe WE, Blazar BR. Sarcoma derived from cultured mesenchymal stem cells. Stem Cells 2007;25:371-379.
260. Torsvik A, Rosland GV, Svendsen A, Molven A, Immervoll H, McCormack E, Lonning PE, Primon M, Sobala E, Tonn JC, Goldbrunner R, Schichor C, Mysliwietz J, Lah TT, Motaln H, Knappskog S, Bjerkvig R. Spontaneous malignant transformation of human mesenchymal stem cells reflects cross-contamination: putting the research field on track - letter. Cancer Res 2010;70:6393-6396.
261. Tronc F, Conter C, Marec-Berard P, Bossard N, Remontet L, Orsini A, Gamondes JP, Louis D. Prognostic factors and long-term results of pulmonary metastasectomy for pediatric histologies. Eur J Cardiothorac Surg 2008;34:1240-1246.
262. Tsukahara T, Kawaguchi S, Torigoe T, Asanuma H, Nakazawa E, Shimozawa K, Nabeta Y, Kimura S, Kaya M, Nagoya S, Wada T, Yamashita T, Sato N. Prognostic significance of HLA class I expression in osteosarcoma defined by anti-pan HLA class I monoclonal antibody, EMR8-5. Cancer Science 2006;97:1374-1380.
263. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proceedings of the National Academy of Sciences of the United States of America 2001;98:5116-5121.
264. Uccelli A, Prockop DJ. Why should mesenchymal stem cells (MSCs) cure autoimmune diseases? Curr Opin Immunol 2010;22:768-774.

265. Uehara H, Shacter E. Auto-oxidation and oligomerization of protein S on the apoptotic cell surface is required for met tyrosine kinase-mediated phagocytosis of apoptotic cells. Journal of Immunology 2008;180:2522-2530.
266. van Dongen M., Savage ND, Jordanova ES, Briaire-de Bruijn IH, Walburg KV, Ottenhoff TH, Hogendoorn PCW, van der Burg SH, Gelderblom H, van HT. Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors. Int J Cancer 2010;127:899-909.
267. Van Ginderachter JA, Movahedi K, Van den BJ, De BP. Macrophages, PPARs, and Cancer. PPAR Res 2008;2008:169414.
268. van Ostaijen-ten Dam M, Verhoeven D, Kraal K, Bongaerts R, van Bergen J, Ball LM, Lankester A, van Tol M, Zwaginga J. Preclinical validation of an NK cell preparation: phenotype and function of NK cell products isolated via CliniMacs and subsequent ex vivo activation with IL-2 or IL-15. Bone Marrow Transplantation 2010;45:S302-S303.
269. Van Rijk-Zwikker GL, Nooy MA, Taminiau A, Kappetein AP, Huysmans HA. Pulmonary Metastasectomy in Patients with Osteosarcoma. European Journal of Cardio-Thoracic Surgery 1991;5:406-409.
270. Velardi A, Ruggeri L, Mancusi A. Killer-cell immunoglobulin-like receptors reactivity and outcome of stem cell transplant. Curr Opin Hematol 2012;19:319-323.
271. Venge P, Bystrom J. Eosinophil cationic protein (ECP). Int J Biochem Cell Biol 1998;30:433-437.
272. Verhoeven DH, de Hooge AS, Mooiman EC, Santos SJ, ten Dam MM, Gelderblom H, Melief CJ, Hogendoorn PCW, Egeler RM, van Tol MJ, Schilham MW, Lankester AC. NK cells recognize and lyse Ewing sarcoma cells through NKG2D and DNAM-1 receptor dependent pathways. Mol Immunol 2008;45:3917-3925.
273. Volodko N, Reiner A, Rudas M, Jakesz R. Tumour-associated macrophages in breast cancer and their prognostic correlations. Breast 1998;7:99-105.
274. Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M. Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis Cartilage 2002;10:199-206.
275. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene 2008;27:5932-5943.
276. Wang LL, Gannavarapu A, Kozinetz CA, Levy ML, Lewis RA, Chintagumpala MM, Ruiz-Maldanado R, Contreras-Ruiz J, Cunniff C, Erickson RP, Lev D, Rogers M, Zackai EH, Plon SE. Association between osteosarcoma and deleterious mutations in the RECQL4 gene in Rothmund-Thomson syndrome. J Natl Cancer Inst 2003;95:669-674.
277. Ward WG, Mikaelian K, Dorey F, Mirra JM, Sassoon A, Holmes EC, Eilber FR, Eckardt JJ. Pulmonary Metastases of Stage Iib Extremity Osteosarcoma and Subsequent Pulmonary Metastases. Journal of Clinical Oncology 1994;12:1849-1858.
278. Whelan J, Patterson D, Perisoglou M, Bielack S, Marina N, Smeland S, Bernstein M. The role of interferons in the treatment of osteosarcoma. Pediatr Blood Cancer 2010;54:350-354.
279. White CD, Brown MD, Sacks DB. IQGAPs in cancer: A family of scaffold proteins underlying tumorigenesis. Febs Letters 2009;583:1817-1824.
280. Wiklund TA, Blomqvist CP, Raty J, Elomaa I, Rissanen P, Miettinen M. Postirradiation sarcoma. Analysis of a nationwide cancer registry material. Cancer 1991;68:524-531.
281. Wunder JS, Gokgoz N, Parkes R, Bull SB, Eskandarian S, Davis AM, Beauchamp CP, Conrad EU, Grimer RJ, Healey JH, Malkin D, Mangham DC, Rock MJ, Bell RS, Andrulis IL. TP53 mutations and outcome in osteosarcoma: a prospective, multicenter study. J Clin Oncol 2005;23:1483-1490.
282. Yamamoto A, Miyazaki T, Kadono Y, Takayanagi H, Miura T, Nishina H, Katada T, Wakabayashi K, Oda H, Nakamura K, Tanaka S. Possible involvement of I kappa B kinase 2 and MKK7 in osteoclastogenesis induced by receptor activator of nuclear factor kappa B ligand. Journal of Bone and Mineral Research 2002;17:612-621.
283. Yang SY, Chen W, Stashenko P, Li YP. Specificity of RGS10A as a key component in the RANKL signaling mechanism for osteoclast differentiation. Journal of Cell Science 2007;120:3362-3371.
284. Yoshimi R, Yamaji S, Suzuki A, Mishima W, Okamura M, Obana T, Matsuda C, Miwa Y, Ohno S, Ishigatsubo Y. The gamma-parvin-integrin-linked kinase complex is critically involved in leukocyte-substrate interaction. Journal of Immunology 2006;176:3611-3624.
285. Zavialov AV, Engstrom A. Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity. Biochemical Journal 2005;391:51-57.
286. Zhou JH, Zhu T, Hu CX, Li HY, Chen G, Xu G, Wang SX, Zhou JF, Ma D. Comparative genomics and function analysis on BI1 family. Computational Biology and Chemistry 2008;32:159-162.

287. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J Leukoc Biol 2007;81:584-592.

288. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nature Reviews Immunology 2008;8:59-73.

289. Benson DM, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, Baiocchi RA, Zhang J, Yu J, Smith MK, Greenfield CN, Porcu P, Devine SM, Rotem-Yehudar R, Lozanski G, Byrd JC, Caligiuri MA. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. Blood 2010; 30:2286-94.

ABBREVIATIONS

ADM	adrenomedullin
ANOVA	analysis of variance
APC	allophycocyanin
AR	antigen retrieval
BSA	bovine serum albumin
CDDP	cis-diamminedichloroplatinum, cisplatin
CT	computed tomography
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAMP	damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DE	differentially expressed
DNA	deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
DX	doxorubicin
E:T	effector:target
EEF1A1	eukaryotic translation elongation factor 1 alpha 1
EOI	European Osteosarcoma Intergroup
EURAMOS	European and American Osteosarcoma Study Group
FBS	fetal bovine serum
FC	flow cytometry
FCS	fetal calf serum
FFPE	formalin-fixed paraffin-embedded
FITC	fluorescein isothiocyanate
grB	granzyme B
HCLS-1	hematopoietic cell specific Lyn substrate 1
HLA	human leukocyte antigen
IF	immunofluorescent
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
JAK	janus kinase
KIR	Killer Immunoglobulin-like Receptor
LIMMA	Linear Models for Microarray Data
mDC	myeloid dendritic cell
MDP	muramyl dipeptide
MFI	mean fluorescence intensity
MHC	major histocompatibility antigen

MIC	major histocompatibility class I polypeptide-related sequence
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MSC	mesenchymal stromal cell
MTP	muramyl tripeptide
MTX	methotrexate
NCR	natural cytotoxicity receptor
NK cell	natural killer cell
NKG2D	Natural Killer Group 2, member D
NOD	nucleotide-binding and oligomerization domain
OS	osteosarcoma
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	population doublings
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PFA	paraformaldehyde
PS	penicillin/streptomycin
PVR	poliovirus receptor
RNA	ribonucleic acid
RT	reverse transcriptase
SAM	Significance Analysis for Microarrays
STAT	signal transducer and activator of transcription
TAM	tumor associated macrophage
TGF-β	transforming growth factor- β
ULBP	UL-16 binding protein
VEGF	vascular endothelial growth factor
WHO	World Health Organisation

PUBLICATIONS

E.P. Buddingh, S.E.N. Ruslan, C.M.A. Reijnders, M.L. Kuijjer, H. Roelofs, P.C.W. Hogendoorn, R.M. Egeler, A.M. Cleton-Jansen, A.C. Lankester. Mesenchymal stromal cells derived from osteosarcoma patients do not transform during long-term culture. *Submitted*.

M.L. Kuijjer, B.E.W.M. van den Akker, R. Hilhorst, M. Mommersteeg, **E.P. Buddingh**, M. Serra, H. Bürger, P.C.W. Hogendoorn, A. M. Cleton-Jansen. Kinome and mRNA expression profiling of high-grade osteosarcoma identifies genomic instability, and reveals Akt as potential target for treatment. *Submitted*.

M.L. Kuijjer, H. Rydbeck, S.H. Kresse, **E.P. Buddingh**, A.B. Lid, H. Roelofs, H. Bürger, O. Myklebost, P.C.W. Hogendoorn, L.A. Meza-Zepeda, A.M. Cleton-Jansen. Identification of osteosarcoma driver genes by integrative analysis of copy number and gene expression data. Genes Chromosomes Cancer. 2012 Jul;51(7):696-706.

E.P. Buddingh, S.E.N. Ruslan, D. Berghuis, H. Gelderblom, J.K. Anninga, P.C.W. Hogendoorn, R.M.Egeler, M.W. Schilham, A.C. Lankester. Intact interferon signaling in peripheral blood leukocytes of high-grade osteosarcoma patients. Cancer Immunol Immunother. 2012 Jun;61(6):941-7.

A.M. Cleton-Jansen, **E.P. Buddingh**, A.C. Lankester. Immunotherapy: Is it different for sarcomas? Oncoimmunology. 2012 Mar 1;1(2):255-257.

D. Berghuis, M.W. Schilham, H.I. Vos, S.J. Santos, S. Kloess, **E.P. Buddingh**, R.M. Egeler, P.C.W. Hogendoorn, A.C. Lankester. Histone deacetylase inhibitors enhance expression of NKG2D ligands in Ewing sarcoma and sensitize for natural killer cell-mediated cytolysis. Clin Sarcoma Res. 2012 Feb 8;2(1):8.

J.H. Pahl JH, S.E.N. Ruslan, **E.P. Buddingh**, S.J. Santos, K. Szuhai, M. Serra, H. Gelderblom, P.C.W. Hogendoorn, R.M. Egeler, M.W. Schilham, A.C. Lankester. Anti-EGFR Antibody Cetuximab Enhances the Cytolytic Activity of Natural Killer Cells toward Osteosarcoma. Clin Cancer Res. 2012 Jan 15;18(2):432-41.

E.P. Buddingh, M.W. Schilham, S.E.N. Ruslan, D. Berghuis, K. Szuhai, J. Suurmond, A.H.M. Taminiau, H.Gelderblom, R.M. Egeler, M. Serra, P.C.W. Hogendoorn, A.C. Lankester. Chemotherapy resistant osteosarcoma is highly susceptible to IL-15 activated allogeneic and autologous NK cells. Cancer Immunol Immunother. 2011 Apr;60(4):575-86.

E.P. Buddingh, M.L. Kuijjer, R. A.J. Duim, H. Bürger, K. Agelopoulos, O.Myklebost, M. Serra, F. Mertens, P.C.W. 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. 2011 Apr 15;17(8):2110-9.

C.M.A. Reijnders, C.J.F. Waaijer, A. Hamilton, **E.P. Buddingh**, S. Dijkstra, J. Ham, E. Bakker, K. Szuhai, M. Karperien, P.C.W. Hogendoorn, S.E. Stringer, J.V.M.G. Bovée. No haploinsufficiency but two hits in EXT in osteochondroma formation. Am J Pathol. 2010 Oct;177(4):1946-57.

E.P. Buddingh, J.K. Anninga, M.I.M. Versteegh, A.H.M. Taminiau, R.M. Egeler, C.S.P. van Rijswijk, P.C.W. Hogendoorn, A.C. Lankester, H. Gelderblom. Prognostic Factors In Pulmonary Metastasized High-Grade Osteosarcoma. Pediatr Blood Cancer. 2010 Feb; 54(2):216-221.

A.B. Mohseny, K. Szuhai, S. Romeo, **E.P. Buddingh**, I. Briaire-de Bruijn, D. de Jong, M. van Pel, A.M. Cleton-Jansen, P.C.W. Hogendoorn. Homozygous Deletion of CDKN2 in Tumorigenic Mesenchymal Stem Cells: a Key Event for Osteosarcoma Genesis. J Pathol. 2009 Nov;219(3):294-305.

A. Taminiau, J. Anninga, H. Gelderblom, C. Rijswijk, P. Hogendoorn, **E. Buddingh**, S. Dijkstra. Chirurgische mogelijkheden bij osteosarcoom: onderdeel van een multidisciplinair probleem. Oncollectie. 2008 (3): 2-7.

E.P. Buddingh, A. van Hylckama Vlieg, F.R. Rosendaal. The Angiotensin-Converting Enzyme Gene insertion/deletion polymorphism: insufficient evidence for a role in deep venous thrombosis. J Thromb Haemost. 2005 Feb; 3 (2): 403-4.

E.P. Buddingh, S. Naumann, M. Nelson, J.R. Neff, N. Birch, J.A. Bridge. Cytogenetic findings in benign cartilaginous neoplasms. Cancer Genetic Cytogenet. 2003 Mar;141(2):164-8.

E.P. Buddingh, P. Krallman, J.R. Neff, M. Nelson, J. Liu, J.A. Bridge. Chromosome 6 abnormalities are recurrent in synovial chondromatosis. Cancer Genet Cytogenet. 2003 Jan;140(1):18-22.

CURRICULUM VITAE

Emilie Pauline (Emmeline) Buddingh'
25 juni 1979, Ede

1990-1997
Middelbare school
Voorbereidend Wetenschappelijk Onderwijs ('90-'96), Radulphus College, Curaçao
Curaçao-American Preparatory School ('96-'97), Curaçao

1997-2004
Geneeskunde, Universiteit Leiden
Artsexamen januari 2005, *Cum Laude*

1999-2004
Wetenschappelijke stages
Pathologie, Moleculaire Cel Biologie, Epidemiologie, LUMC, Leiden
Klinische genetica, VUMC, Amsterdam
Pathologie, University of Nebraska Medical Center, Nebraska, VS

2004
Keuze-coschappen
Klinische genetica, VUMC, Amsterdam
Tropengeneeskunde, Igogwe Hospital, Tukuyu, Tanzania

2005
Arts-assistent niet in opleiding
Kindergeneeskunde, Het LangeLand ziekenhuis, Zoetermeer

2006
Arts-assistent niet in opleiding
Kindergeneeskunde, Sophia Kinderziekenhuis, ErasmusMC, Rotterdam

2007 - 2010
Promotie-onderzoek (ZonMW AIOSKO-beurs)
Immunologisch Laboratorium Kindergeneeskunde
& afdeling Pathologie, LUMC, Leiden

Sinds 2010
Kinderarts in opleiding
Willem-Alexander Kinderziekenhuis, LUMC, Leiden
& Juliana Kinderziekenhuis, Hagaziekenhuis, Den Haag

DANKWOORD

In de aanloop van het project, gedurende de drie labjaren en in de afrondingsfase daarna ben ik door velen geholpen. Ik hoop dat ik gaandeweg mijn waardering al heb laten blijken, maar ik maak toch graag van de gelegenheid gebruik om een aantal mensen met nadruk te bedanken.

Dr. A.C. Lankester, co-promotor. Arjan, fundamenteel onderzoek en klinisch werk gaan bij jou hand in hand, dat is een mooi voorbeeld dat ik ooit hoop te kunnen volgen. Dank voor de vrijheid die je me gaf om zelf (mede) de richting van het onderzoek te bepalen en in de afronding mijn eigen tempo te kiezen. Prof. Dr. P.C.W. Hogendoorn, promotor. Pancras, onder jouw vleugels zette ik als tweedejaars geneeskundestudent de eerste schreden in onderzoeksland. Ik vind het erg leuk dat jij niet alleen aan het begin van mijn onderzoekservaring een sturende rol hebt gehad, maar dat ook in opzet, uitvoering en voltooiing van deze promotie hebt gehad. Prof. Dr. R.M. Egeler, promotor. Maarten, in alle fasen van dit onderzoek heb jij me steeds met positieve bijdragen ondersteund. Ook vanuit Toronto waren jouw commentaren op de laatste onderdelen van dit proefschrift steeds bemoedigend, dank voor deze steun in de rug tijdens de laatste loodjes.

Dr. Cleton-Jansen en Dr. Schilham: Anne-Marie en Marco, jullie hebben beiden ontzettend veel betekend voor dit proefschrift. Marco, je kritische blik hebben veel dingen aangescherpt en beter gemaakt. Ettelijke keren waaide ik onaangekondigd met een vraag je kamer in en kon jij weer een lacune in mijn immunologie-kennis dichten, dank daarvoor. Anne-Marie, ik denk nog met veel plezier terug aan die keer dat jij letterlijk springend naast de microscoop stond toen we een binucleaire MSC hadden ontdekt. Dank voor je enthousiasme en positivisme.

Mijn lieve maatjes in het lab, Dagmar Berghuis en Willemijn Quispel, dank voor alle gezelligheid en steun, toen en nu! Wat fijn dat jullie mijn paranimfen willen zijn. Dagmar, niemand anders weet zó goed hoe het is een proefschrift over sarcoom-immunologie te verdedigen, het is een geruststellend idee dat jij tijdens de verdediging naast mij staat. Willemijn, jouw kritische blik en overdosis empathie waardeerde ik eerst als collega, waardeer ik nu als vriendin en maakt van jou straks ongetwijfeld een geweldige arts.

Alle lab-collega's van de kindergeneeskunde en de pathologie, bedankt voor de gezelligheid, collegialiteit en waar nodig praktische hulp. Riri, you are a very fun person to be around and I am honoured and grateful that you were the technician on this project. Susy, mede dankzij je Zuid-Amerikaanse roots en je warme karakter heb ik me snel thuis gevoeld op het lab, muito obrigada! Mijn studenten Bas, Kimberly, Sharon, Joyce, Milou en Jolien: hoewel niet al jullie bijdragen de weg naar dit proefschrift gevonden hebben, heb ik van elk van jullie wat geleerd, dank daarvoor en dank voor jullie inzet. Alle mede-aio's en P3-35 kamergenoten: dank voor de samenwerking en de fijne tijd. Alle huidige en voormalige collega's van de kindergeneeskunde in Leiden en Den Haag, dank voor de interesse en de bemoedigende woorden tijdens de afrondingsfase.

Speciale dank gaat uit naar de patiënten en hun ouders. Juist op het moment van het krijgen van een verpletterende diagnose waren er zovelen die onbaatzuchtig 'ja' zeiden op de vraag of ze mee wilden doen aan wetenschappelijk onderzoek. Zonder deze bijdrage waren de meeste onderdelen van dit proefschrift niet mogelijk geweest. Ook dank ik iedereen die heeft geholpen

met het verkrijgen van informed consent en het verzamelen en verwerken van de biologische materialen, in het bijzonder Professor Hans Gelderblom, Professor Antonie Taminiau, Sander Dijkstra, Nicolette Leyerzapf, Karoly Szuhai, Daniëlle de Jong en Jakob Anninga.

Familie en vrienden, hier of op een zeker tropisch eiland, dank voor de broodnodige ontspanning naast het werk. Dank voor alle leuke weekenden, etentjes, vakanties, borrels en BBQs. Met name Juliëtte, Donnée, Liesbeth, Sandra en Marlies: dank!

Lieve ouders, goed voorbeeld doet goed volgen: jullie plezier en vasthoudendheid in het doen van onderzoek en het schrijven van boeken is inspirerend. Dit, en jullie onvoorwaardelijke steun, heeft zeker bijgedragen aan het voltooien van dit proefschrift.

Lieve Maarten, dank voor alles! Als arts zijn er grote hiaten in mijn moleculair biologische kennis, die jij onvermoeibaar opvult. Maar bovenal duizendmaal dank voor je steun en liefde, in voorspoedige en minder voorspoedige tijden. Wat bof ik.