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Human Cancer Biology

Clinical Cancer Research

Tumor-Infiltrating Macrophages Are Associated with Metastasis Suppression in High-Grade Osteosarcoma: A Rationale for Treatment with Macrophage Activating Agents

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Abstract

Purpose: High-grade osteosarcoma is a malignant primary bone tumor with a peak incidence in adolescence. Overall survival (OS) of patients with resectable metastatic disease is approximately 20%. 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 carried out genome-wide gene expression profiling on prechemotherapy biopsies of patients who did ($n = 34$) and patients who did not ($n = 19$) develop metastases within 5 years. Immunohistochemistry (IHC) was performed on pretreatment biopsies from 2 additional cohorts ($n = 63$ and $n = 16$) and corresponding postchemotherapy resections and metastases.

Results: A total of 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 (TAM) were quantified with IHC and associated with significantly better overall survival (OS) 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).

Conclusions: 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. *Clin Cancer Res; 17(8); 2110–9. ©2011 AACR.*

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 older than 40 years (1). Despite wide-margin surgery and intensification of chemotherapeutic treatment, overall survival (OS) rates have reached a plateau at about 60% (2–4). Novel administration 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 IFN-α in patients with good histological response to neoadjuvant chemotherapy (5). A recently published clinical trial has shown improved OS for osteosarcoma patients treated with the macrophage activating agent muramyl tripeptide (MTP) added to the standard chemotherapy regimen (6). However, only limited information on macrophage infiltration and activation in osteosarcoma is available (7).

Tumor-associated macrophages (TAM) may promote tumorigenesis through immunosuppression, expression of matrix-degrading proteins and support of angiogenesis.

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Translational Relevance

We have shown by genome-wide mRNA profiling and immunohistochemistry that the presence of macrophages in primary prechemotherapy biopsies is associated with suppression of metastasis and thus survival in several cohorts of osteosarcoma. Osteosarcoma is a primary bone tumor, affecting especially adolescents. Survival has not improved since the introduction of adjuvant chemotherapy. Our findings support the use of liposomal muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) as an adjuvant drug in treatment of osteosarcoma and provides a biological rationale for clinical efficacy of this compound, because L-MTP-PE is an effective activator of macrophages. L-MTP-PE has recently been approved by EMEA and our data provide an important biological background that can guide future clinical decisions regarding the implementation of macrophage activating agents in osteosarcoma adjuvant treatment regimens.

In numerous cancer types, high numbers of M2 or "alternatively activated" TAMs are associated with a worse prognosis (8–13). M2 macrophages have important functions in wound healing and angiogenesis, express high levels of the immunosuppressive cytokines interleukin (IL)-10 and TGF-β, and express scavenger receptors such as CD163 (14, 15). "Classical activation" of macrophages by IFN-γ or microbial products results in polarization toward M1-type macrophages. M1 macrophages express high levels of proinflammatory cytokines such as IL-12, IL-1, and IL-6 and have potent antitumor efficacy, both by reactive oxygen species and cytokine-induced cytotoxicity and by induction of natural killer (NK) and T cell activity (16). Rarely, high numbers of TAMs are associated with better prognosis (17, 18). In these cases, TAMs are presumably polarized toward an M1 phenotype, although macrophage subtypes were not reported in these 2 studies. Alternatively, macrophages may directly phagocytose tumor cells, as has been shown in acute myeloid leukemia (19).

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. By using genome-wide expression analysis, we showed that high expression of macrophage-associated genes in pretreatment biopsies was associated with a lower risk of developing metastases. In addition, we quantified and characterized TAMs in 2 independent cohorts, including pretreatment biopsies, postchemotherapy 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 pretreatment 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 pretreatment biopsies, postchemotherapy resections, and metastatic lesions (20). Clinicopathological details can be found in Supplemental Table S1. 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 19 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) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Invitrogen) as previously described (21).

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) and stored at −70°C. By 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 with TRIzol (Invitrogen), followed by RNA cleanup using the QIAGEN Rneasy mini kit with on-column DNase treatment. RNA quality and concentration were measured using an Agilent 2100 Bioanalyzer and Nanodrop ND-1000 (Thermo Fisher Scientific), respectively. Synthesis of cDNA, cRNA amplification, and hybridization of cRNA onto the Illumina Human-6 v2.0 Expression BeadChips was carried out as per manufacturer's instructions.

Reverse transcriptase quantative PCR

Reverse transcriptase quantative PCR (RT-qPCR) analysis of selected target genes was performed as previously described (22). Each experiment was conducted in duplicate by using an automated liquid-handling system (Tecan, Genesis RSP 100). Data were normalized by geometric mean expression levels of 3 reference genes, i.e., *SRPR*, *CAPNS1*, and *TBP* using geNorm (http://medgen.ugent.be/~jvdesomp/genorm/). Primer sequences can be found in Supplemental Table S2.

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Enzymatic and fluorescent immunostainings

Enzymatic and fluorescent immunostainings were performed on 4 μm sections of FFPE tissue as previously described (20). Details regarding antibodies and procedures can be found in Supplemental Table S3. In case of double immunohistochemistry (IHC), incubation with anti-CD45 and development with DAB+ (Dako) 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). In case of double immunofluorescent (IF) stainings, primary antibodies were coincubated overnight. As a positive control, normal and formic acid decalcified tonsil was used, and 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). Numbers of positively stained cells and vessels were counted using ImageJ (National Institutes of Health, Bethesda, MD) and averaged per 0.6 mm core. IF and double IHC images were acquired using a Leica DM4000B microscope fitted with a CRI Nuance spectral analyzer (Cambridge Research and Instrumentation, Inc.) and analyzed using the supplied colocalization tool to determine the 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 (23). As Illumina identifiers are not stable and consistent between different chip versions, raw oligonucleotide sequences were converted to nuIDs (24). Data were transformed using the variance stabilizing transformation algorithm to take advantage of the large number of technical replicates available on the Illumina BeadChips (25). 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 by Bioconductor package lumi (26, 27). Quality control was performed using Bioconductor package arrayQualityMetrics (28). MIAME (minimum information about a microarray experiment) 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 5 years from diagnosis of the primary tumor was determined using linear models for microarray data (LIMMA; ref. 29), applying a Benjamini and Hochberg false discovery rate–adjusted *P*-value cutoff of 0.05. Other univariate statistical analyses were performed using GraphPad Prism Software (version 5.01). Multivariate survival analyses were carried out according to the Cox proportional hazards model in SPSS (version 16.0.2). 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 5 years from diagnosis (cohort 1)

Comparison of genome-wide gene expression in tumors of patients who did and did not develop metastases within 5 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 1 and Supplemental Table S4, 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 that 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 RT-qPCR correlated well with expression levels obtained by microarray analysis (Supplemental Fig. S1). Metastases-free survival curves of the same cohort, generated using median expression of the probe of interest as a cutoff determining low and high expression, are shown in Figure 1B and Supplemental Figure 2. Cox proportional hazards analysis revealed expression of macrophage-associated genes *CD14* and *HLA-DRA* to be independently associated with metastasis-free survival (Supplemental Table S5).

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

The most probable source of expression of the DE macrophage–associated genes was 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 because only osteosarcoma biopsies contain macrophage infiltrate, whereas RNA from cell lines is exclusively from tumor cells (Fig. 1A, Mann-Whitney U test $P < 0.0001$). 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. 1C). We chose this

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Table 1. DE genes and probes by category comparing high-grade osteosarcoma patients with and without metastases within 5 years by genome-wide expression profiling (cohort 1)

Category	Higher expression in patients without metastases			Lower expression in patients without metastases		
	Number of probes	Number of genes	Examples	Number of probes	Number 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, HLA-DQA1*	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	

Note: Twenty percent of DE probes corresponded to genes that 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.

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*.

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. 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, $P = 0.01$). Similar to the gene expression data, there was a trend for patients with primary localized disease to have higher numbers of macrophages in pretreatment 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

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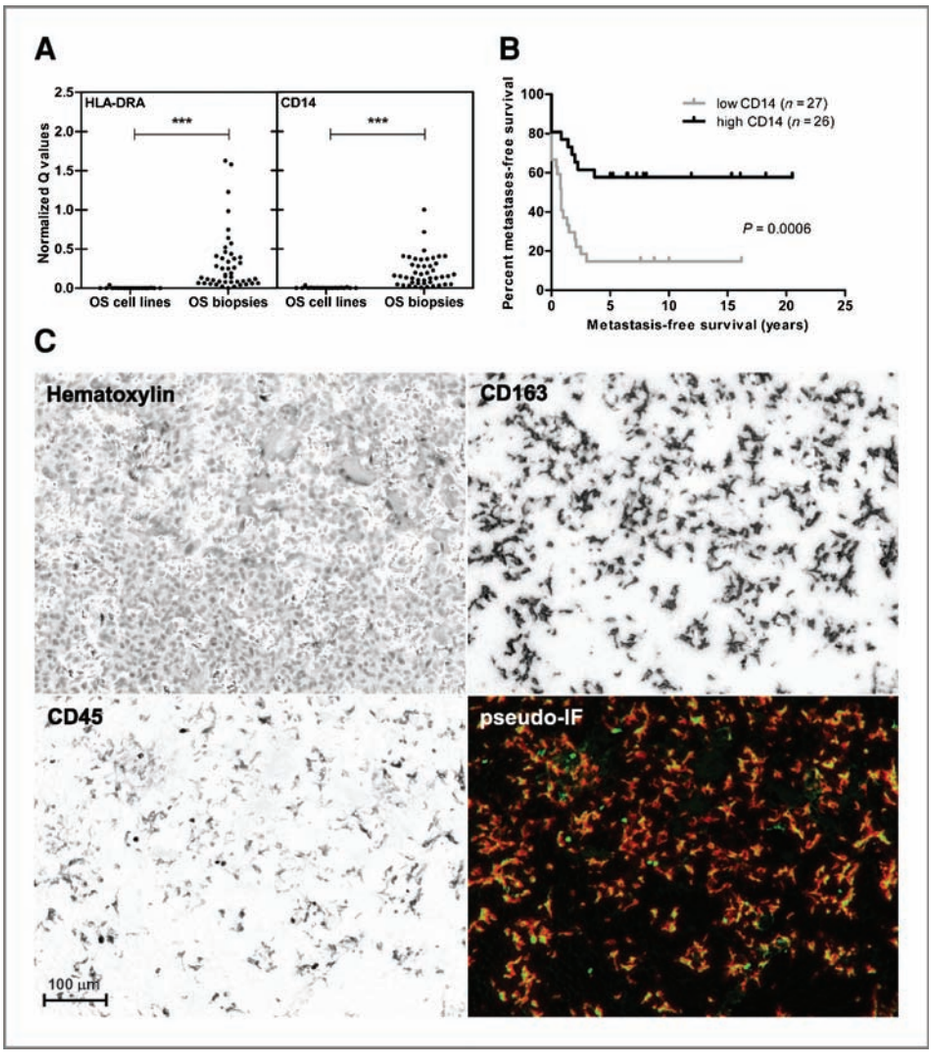

Figure 1. Macrophage-associated genes are not expressed by osteosarcoma tumor cells. A, RT-qPCR of osteosarcoma cell lines and biopsies of *CD14* and *HLA-DRA* demonstrating lack of expression by osteosarcoma cells. Mann-Whitney U test $P < 0.0001$, ***. B, high expression of macrophage associated genes was associated with a better metastasis-free survival (cohort 1, Kaplan–Meier curve, *P* value obtained by log-rank 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 the time of diagnosis). Metastasis-free survival curves for *HLA-DRA, CLEC5A*, and *FCGR2A* can be found in Supplemental Figure S2. C, double immunohistochemical staining of CD163 with the hematopoietic cell marker CD45 was performed and analyzed using spectral imaging microscopy. The pseudo-IF image (pseudo-IF) shows CD163-positive cells in red, CD45-positive cells in green, and colocalization 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.

high macrophage counts at diagnosis tended to be less likely to develop metastases within 5 years (χ^2, $P = 0.13$).

We subdivided this cohort into 4 quartiles based on numbers of CD14-positive cells to determine the group with the best OS. No significant differences were found between quartiles 2 and 4, but patients belonging to this group had better OS than patients with low CD14 counts (lowest quartile, or less than 12 CD14-positive cells per tissue array core; Fig. 2B, log-rank test $P = 0.02$). 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. 3, log-rank test $P = 0.01$, Supplemental Fig. S3).

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. 3A and Supplementary Fig. S3A). The total number of macrophages as determined by quantifying CD14-positive macrophages was associated with good survival (Fig. 3B), but the phenotype of the macrophages (CD14/CD163 double positive versus CD14/HLA-DRα double positive) was not (Supplemental Fig. S3B; data not shown). Another M2 characteristic is support of angiogenesis. The number of CD14-positive macrophages correlated with the number of CD31-positive vessels (Figs. 2A and 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 3 quartiles or more than 12 CD14-positive cells per tissue

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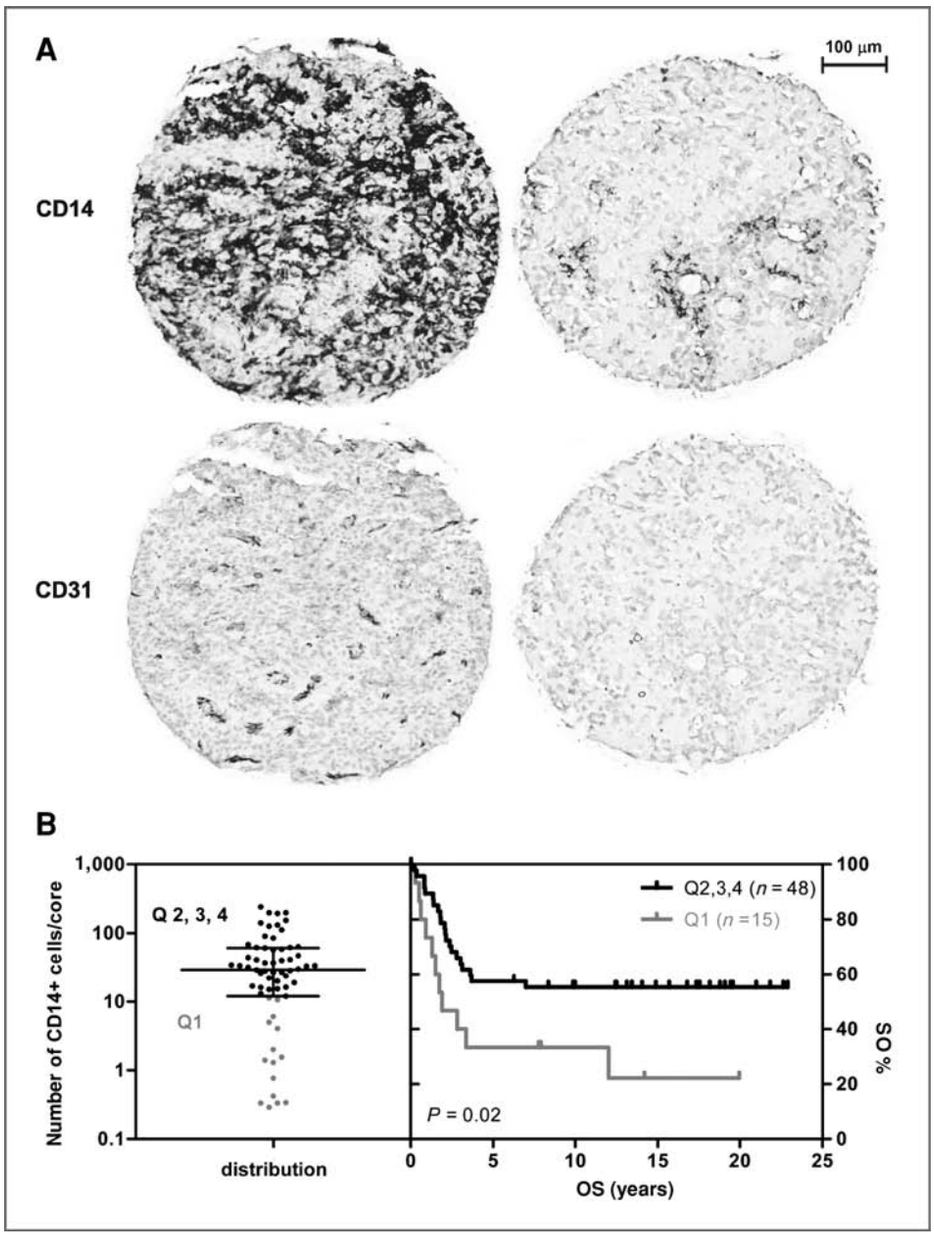

Figure 2. A, example of representative stainings of high-grade osteosarcoma with high (left) versus low (right) levels of macrophage infiltration (CD14 staining) and vascular density (CD31 staining). B, high numbers of infiltrating macrophages (left, defined as the 3 upper quartiles, or more than 12 CD14-positive cells per tissue array core) are associated with better OS (right, log-rank test $P = 0.02$, cohort 2). Q1, lowest quartile; Q2, 3, 4, 3 highest quartiles.

array core) in prechemotherapy diagnostic biopsies of the primary tumor to predict for good histological response to neoadjuvant chemotherapy (defined as more than 90% nonvital tumor tissue upon final resection), since 46% of patients with high macrophage numbers and 18% of patients with low macrophage numbers had a good histological response (cohort 2; $\chi^2 = 0.09$). The prognostic benefit of macrophage counts in osteosarcoma was not independent of histological response using Cox proportional hazard analysis. Macrophage numbers were higher in postchemotherapy resections of the primary tumor than in prechemotherapy biopsies (Supplemental Fig. S4). Moreover, gene expression analysis showed upregulation of macrophage-associated probes in postchemotherapy resections ($n = 4$) as compared with prechemotherapy biopsies ($n = 79$, data not shown).

Discussion

OS of high-grade osteosarcoma patients with resectable metastatic disease is poor at about 20% (30). 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

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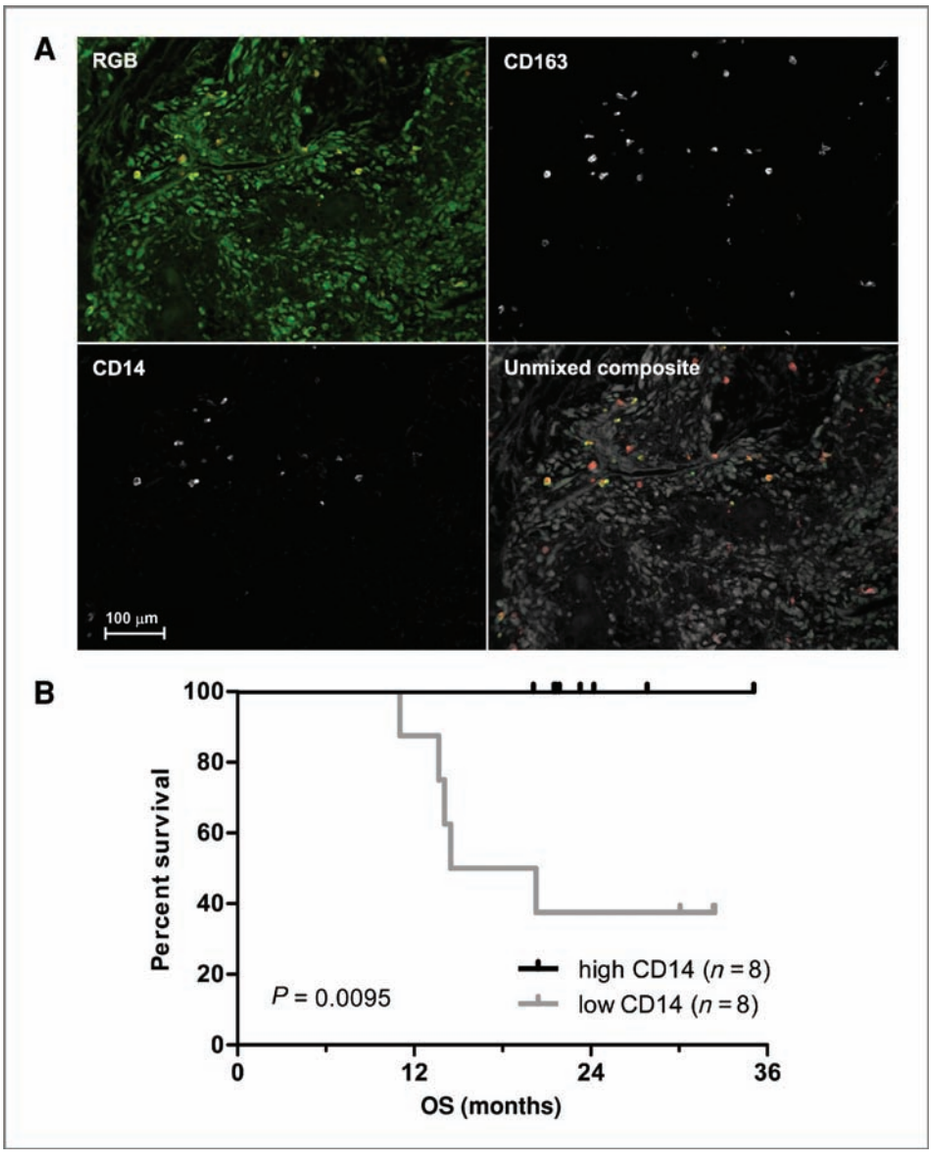

Figure 3. A, osteosarcoma samples are infiltrated with CD14 and CD163 single and double positive macrophages. Spectral imaging was used to reduce autofluorescence of osteosarcoma cells. In the composite image, CD14-positive cells are represented in green, CD163-positive cells are represented in red, and CD14/CD163 double positive cells are represented in yellow. Background autofluorescence of tumor cells is represented in gray. B, in an independent cohort of 16 patients (cohort 3), high macrophage infiltration as determined by IF CD14 staining was associated with significantly improved OS. *P* values obtained using log-rank test, cutoff at the median.

prechemotherapy biopsies of osteosarcoma patients. We compared patients who developed clinically detectable metastases within 5 years with patients who did not develop metastases within this time frame (cohort 1). About 20% of genes overexpressed in patients without metastases were macrophage-associated, whereas an additional 25% of genes had other immunological functions (e.g., in phagocytosis, complement activation or cytokine production and response) but could still be attributed to macrophages (Table 1 and Supplemental Table S4). Thus, in total, almost half of the DE genes belonged to 1 specific process, i.e., macrophage function. Macrophage-associated genes were expressed by infiltrating hematopoietic cells and not by osteosarcoma tumor cells (Fig. 1), indicating a possible role for macrophages in preventing metastasis in osteosarcoma. To confirm these findings, we quantified infiltrating macrophages in 2 additional cohorts (cohorts 2 and 3) and found an association with better OS in both cohorts.

The antimetastatic effect of TAMs in osteosarcoma is remarkable because 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 (31). 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 antitumor activity of macrophages apparently outweighs their possible

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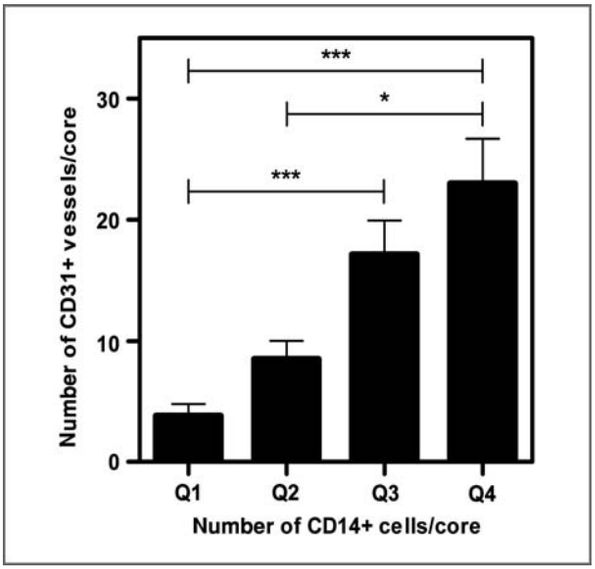

Figure 4. Macrophage infiltration as determined by CD14-positive cell count correlated with vascularity as determined by CD31-positive vessel count. Data of all osteosarcoma samples (pre- and posttreatment primary tumor and metastatic samples, cohort 2) are shown. Q1, lowest quartile; Q2, 3, 4, 3 highest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

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 (32–34). 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 (31, 35). Some of the DE genes, such as *MSR1* and *MS4A6A* are specific for M2 macrophages *in vitro* (36). Others, such as the proinflammatory cytokine *IL1B*, are more indicative of an M1 phenotype (16). How macrophages inhibit osteosarcoma metastasis and whether 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 (37, 38). The binding of these dangerous 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 antimetastatic effect of macrophages. Perhaps, the antimetastatic 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 6-year OS from 70% to 78% in a cohort of patients with primary localized disease (6, 39). Similar results were obtained in canine osteosarcoma (40). 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 [NOD-like receptor (NLR)] family, expressed by macrophages (41). In our study, 5 genes associated with NLR family signaling and the associated "inflammasome" were highly expressed in pretreatment biopsies of patients who do not develop metastases. The DE genes *NLRP3*, *NAIP*, *NLRC4*, and *PYCARD* are components of the inflammasome, *LYZ* is a lysozyme that processes bacterial cell wall peptidoglycan into MDP, a ubiquitous natural analogue of MTP, and *IL1B* is the downstream effector cytokine of the inflammasome pathway. Further research is needed to clarify whether only patients with high numbers of TAMs benefit from MTP treatment, or whether MTP treatment is effective regardless of macrophage number or activation status pretreatment. Also, it is unknown whether treatment with agents promoting macrophage migration or with other macrophage activating agents like toll-like receptor ligands or IFNs has a similar beneficial effect on outcome.

Previous genome-wide expression profiling studies in osteosarcoma focused on identifying genes that predict histological response to neoadjuvant chemotherapy (42–45). 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 (46). There is a 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 DE group of genes and the macrophage-associated genes *MSR1*, *MS4A6A*, and *FCGR2A*).

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

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Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflicts of interest.

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