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Association of Macrophage and Lymphocyte Infiltration with Outcome in Canine Osteosarcoma

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Abstract

Immunotherapeutic strategies have shown promise for the treatment of canine osteosarcoma (cOSA). Very little is known about the immune microenvironment within cOSA however, limiting our ability to identify potential immune targets and biomarkers of therapeutic response. We therefore prospectively assessed the disease-free interval (DFI) and overall survival time (ST) of

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Conflict of Interest

The authors have no conflicts of interest in regards to the content of this manuscript.

30 dogs with cOSA treated with amputation and 6 doses of adjuvant carboplatin. We then quantified lymphocytic (CD3+, FOXP3+) and macrophage (CD204+) infiltrates within the primary tumors of this cohort using immunohistochemistry, and evaluated their association with outcome. Overall, the median DFI and ST were 392 and 455 days, respectively. The median number of CD3+ and FOXP3+ infiltrates were 45.8 cells/mm^2 (4.6 – 607.6 cells/mm^2) and 8.5 /mm^2 (0 – 163.1 cells/mm^2), respectively. The median area of CD204+ macrophages was 4.7% (1.3% - 23.3%), and dogs with tumors containing greater than 4.7% CD204+ macrophages experienced a significantly longer DFI (P = 0.016). Interestingly, a significantly lower percentage of CD204+ macrophages was detected in cOSA arising from the proximal humerus compared to other appendicular bone locations (P = 0.016). Lymphocytic infiltrates did not appear to correlate with outcome in cOSA. Overall, our findings suggest macrophages may play a role in inhibiting cOSA progression, as has been suggested in human osteosarcoma.

Keywords

Dogs; immunotherapy; macrophages; osteosarcoma; tumor microenvironment

1. Introduction

Canine osteosarcoma (OSA; cOSA) is a highly metastatic cancer with stagnant survival rates over the last 30 years.[1–3] Broadly, the best patient outcomes have been reported in dogs receiving amputation followed by platinum chemotherapy, with or without the addition of doxorubicin, however the optimal chemotherapeutic protocol has not been precisely determined.[1–16] Unfortunately, two-year survival rates remain around 20% and the majority of dogs still die from pulmonary metastases, warranting investigation into alternate strategies such as immunotherapy to improve survival.[1–8]

In an attempt to improve upon survival estimates for dogs with cOSA receiving standard cytotoxic chemotherapy regimens, several reports have described the addition of immune-targeting therapeutics. Firstly, the finding of limb-sparing associated wound infections as being a positive prognostic factor suggested innate immune responses may be important in delaying or preventing metastatic disease.[17,18] Indeed, this observation was mechanistically supported by a subsequent study showing the presence of macrophages and natural killer cells were essential for mediating the effect of wound infection on decreasing pulmonary metastases in a mouse model of OSA.[19] Furthermore, early studies recognized improved survival in dogs with cOSA treated with amputation followed by liposomal muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) alone compared to empty liposomes, or in a schedule-dependent combination with cisplatin compared to cisplatin alone.[20,21] Interestingly, the anti-cancer activity of L-MTP-PE is also thought to be predominantly due to its ability to promote tumoricidal activity of monocytes and macrophages.[22] Meanwhile, immunotherapeutics predominantly targeting the lymphocytic response have also been utilized with some success. For example Khanna et al. reported complete remissions in 2 of 4 dogs with grossly metastatic cOSA treated with inhaled interleukin-2 (IL-2) liposomes.[23] More recently, Mason et al. described improved survival rates in dogs additionally treated with a HER2-targeting *Listeria* vaccine compared to dogs treated with amputation and

carboplatin alone.[24] Collectively, these studies illustrate the therapeutic potential of activating both innate and adaptive components of the immune response in dogs with cOSA.

While there are several examples of the potential for immune system mobilization to treat cOSA, very little is known about the immune microenvironment within the tumor. An understanding of the type and extent of immune cell infiltration in cOSA is necessary to elucidate potential therapeutic targets, as well as develop predictive and prognostic biomarkers. Prior studies have predominantly focused on the lymphocytic infiltrates in cOSA. For example, Biller et al. identified decreased blood CD8/T regulatory (Treg) ratio as a negative prognostic factor in 12 dogs with cOSA treated with amputation and carboplatin-based chemotherapy, although differences in outcome between dogs with differing intratumoral CD8/Treg ratio did not reach significance.[25] Furthermore, Modiano et al. reported that post-treatment infiltrating lymphocytes were an indicator of response to Fas-ligand gene therapy.[26] Paired pretreatment biopsies were not available in the majority of dogs however, making it unclear whether the inherent immunogenicity of some cOSA might affect outcome independent of the therapeutic intervention. Meanwhile, in human OSA (hOSA) several studies have shown elevations of tumor-infiltrating macrophage populations to be at least as important as lymphocyte populations in predicting a positive response to treatment.[27–29] In peripheral blood, increased circulating myeloid-derived suppressor cells (MDSCs), and decreased expression of chemokine receptors and impaired chemotactic function of monocytes are observed dogs with cOSA compared to healthy controls.[30–32] Furthermore, elevated blood monocyte and lymphocyte counts have been associated with a worse prognosis in cOSA.[33] While peripheral blood indicators of inflammation unquestionably have overall prognostic significance, some studies suggest discordance between indicators of immune response in the periphery and tumor response to immunotherapeutics.[34] Furthermore, the peripheral blood immune landscape frequently is disassociated from that within the tumor.[35–38] While tumor samples are frequently more challenging to obtain than peripheral blood, direct evaluation of the intratumoral immune microenvironment is necessary to understand host-tumor interactions.[39]

Recent advancements in our understanding of cancer immunology have revealed the intratumoral immune microenvironment is a critical determinant of response to various strategies of immune system mobilization. Presently, the best example of immune microenvironment biomarkers guiding therapeutic intervention is the detection of elevated programmed death-ligand 1 (PD-L1) expression by tumors more likely to respond to programmed death-1 (PD-1)/PD-L1 inhibition.[40,41] In several tumor types, including hOSA, PD-L1 expression is positively correlated with the amount of tumor-infiltrating lymphocytes (TILs), indicating that TILs may be a suitable surrogate biomarker for response to checkpoint inhibition.[42–46] Furthermore, hOSA infiltration by a high number of CD163+ macrophages is associated with an improved prognosis in humans treated with standard cytotoxic chemotherapy.[29] Additional treatment with zoledronate however, which is known to modulate macrophage activity, negated the survival advantage associated with elevation of this macrophage population.[29] This study also identified elevated CD8+ lymphocytes within the tumor as being associated with an improved survival in zoledronate treated patients, and proposed an algorithm to guide the rational implementation of immunotherapies based on pretreatment tumor immunoscores. These studies suggest a thorough understanding of the

intratumoral immune microenvironment has the potential to elucidate biomarkers that can guide therapeutic strategies, however neither infiltrating lymphocytes nor macrophages have been quantified and correlated with outcome in standardly treated cOSA to date.

The aim of this study was therefore to evaluate the possible association of immune cell tumor infiltrates (T lymphocytes, macrophages, and FOXP3 expressing cells) with outcome in a prospective cohort of dogs treated with amputation and 6 doses of carboplatin chemotherapy. Given reports in hOSA that generally describe the positive effect of an array of different macrophage subsets on outcome, the authors hypothesized that dogs with high intra-tumoral CD204+ macrophage infiltration of their primary cOSA would experience a prolonged survival when compared to those with low CD204+ macrophage infiltration.

2. Methods

2.1 Prospective clinical evaluation

Dogs with histologically confirmed appendicular OSA whose owners elected adjuvant chemotherapy consisting of 6 doses of carboplatin were prospectively studied. For inclusion, dogs had to have undergone amputation with complete excision of their tumor, no gross metastasis on thoracic radiographs, and started chemotherapy within 1 month of amputation. All treatments were administered at the University of California, Davis William R. Pritchard Veterinary Medical Teaching Hospital (VMTH).

Dogs were prescribed 6 doses of carboplatin at a starting dose of 300 mg/m^2 and a 21-day interval. Dose adjustments and delays due to gastrointestinal toxicity or myelosuppression were allowed at the discretion of the treating clinician. Concurrent chemotherapy, radiation therapy, or tyrosine kinase inhibitor therapy were not allowed, but no other specific medications, including NSAIDS, were disallowed. Routine monitoring for pulmonary metastasis with thoracic radiographs occurred during therapy at the time of the third chemotherapy dose, at the time of the sixth chemotherapy dose, and every 3 months thereafter. Dogs presenting between routine recheck appointments had additional thoracic and/or bone radiographs performed if clinical signs suspicious for metastasis were present. All radiographs were taken at the VMTH and were reviewed by a board certified radiologist. Rescue therapy of any kind was allowed once metastasis was documented.

Data collected for each dog included signalment, weight, tumor location, histologic subtype, mitoses per 3 high power fields (hpf), pre-surgical serum alkaline phosphatase (ALP), time between amputation and the first dose of chemotherapy, dose reductions, treatment delays, outcome, cause of death, and necropsy information if available. Mitoses per 3 hpf were classified as greater than 5 vs 5 or less. Pre-surgical ALP was recorded only if run less than 1 month prior to amputation and results were classified as normal or elevated based on the reference range for the laboratory running the test.

2.2 Tissue collection and processing

Archived formalin-fixed paraffin-embedded (FFPE) blocks of tissue collected from primary tumors immediately after amputation were retrieved and cut into 5μm sections. Decalcified blocks were recorded. At least 1 slide from each tumor was stained with hematoxylin and

eosin (H&E) and the remainder were adhered to charged slides in preparation for immunohistochemistry (IHC).

2.3 IHC processing

Slides underwent standard methods of deparaffinization and rehydration by bathing slides in a xylene alternative (Histo-Clear; National Diagnostics; Atlanta, GA, USA), and graded concentrations of ethanol (Koptec; King of Prussia, PA, USA) in water. Endogenous peroxidase activity was suppressed by incubating slides with 0.09% hydrogen peroxide (Fisher Scientific; Fair Lawn, NJ, USA) in phosphate buffered saline (PBS, Corning, NY, USA) for 25 minutes. Antigen retrieval was performed in pre-heated citrate buffer (H-3300, Vector; Burlingame, CA, USA) in a water bath at >95°C for 25 minutes. Samples were blocked with 2.5% normal goat serum (Vector; Burlingame, CA, USA) for 20 minutes, followed by 5% non-fat milk (Lab Scientific; Highlands, NJ, USA) for 30 minutes at room temperature. Primary antibodies were diluted in Dako Antibody Diluent (CD3, CD204; Dako; Carpinteria, CA, USA) or Signal Stain Ab Diluent (FOXP3; Cell Signaling; Danvers, MA, USA), added to the slides, and incubated overnight at 4°C. Anti-CD3 (rat anti-human, CD3–12; Moore Lab; Davis, CA, USA) was diluted at 1:50, anti-FOXP3 (rat anti-mouse/rat, FJK-16S; Thermo Fisher Scientific; San Diego, CA, USA) was diluted at 1:25, and anti-CD204 (mouse anti-human, SRA-E5; Cosmo Bio; Carlsbad, CA, USA) was diluted at 1:400. Anti-CD3 and –FOXP3 clones are marketed as cross-reactive in canine tissue by their manufactures, while anti-CD204 has shown canine cross-reactivity in several previous studies.[47,48] The ImmPRESS™ horseradish peroxidase (HRP) polymer detection kit (anti-mouse IgG, MP-7452; anti-rat IgG, MP-7444; Vector; Burlingame, CA, USA) was then used as a secondary stain by incubating slides for 30 minutes at room temperature. HRP substrate (Vector NovaRed™ peroxidase substrate kit; Vector; Burlingame, CA, USA) was subsequently applied for 4 minutes. Slides were then counterstained in hematoxylin (Dako; Carpinteria, CA, USA), dehydrated in graded concentrations of ethanol and xylene alternative, and had cover slips applied (Permount, Fisher; Fair Lawn, NJ, USA).

Sections of normal canine lymph node were used as positive and negative controls for CD3, FoxP3 and CD204 staining by evaluating the distribution of their staining within the lymph node. If slides from additional samples were stained subsequently to the initial set, one slide from the first run was stained again in the second run to ensure consistent staining between batches of IHC.

2.4 IHC quantification

Three cellular 100× fields on H&E stained slides from each tumor were selected to mimic previous studies in human OSA where 3 cellular 1–2mm diameter areas within each block were chosen to create tissue microarrays.[27,29,49,50]. The scale was set using an image of a hemocytometer and Image J 1.51s (National Institutes of Health, USA), and determined to be 2041.8 pixels/mm at 100× magnification. The area of each 100× magnification image equaled 1.2mm^2. These areas were marked and transposed onto IHC stained slides from each tumor so that similar areas of tissue were evaluated on subsequent slides for each marker evaluated. A Leica DM2000 was used to image each 100× field, and images were digitalized using the attached Jenoptik ProgRes® C5 camera.

To quantify CD3 and FOXP3 cellular infiltrates, Image J was used to measure the cellular area of each 100× image, and to count the number of positive cells within that area. The area in pixels was then converted to mm^2, and the number of positive cells/mm^2 of cellular area was calculated. Since macrophages were abundant within these tumors, the percent area of CD204+ staining (%CD204+) within each cellular area was determined using Image J, as previously described.[51] Briefly, the cellular area was measured (Suppl. Fig. 1A). Then, images were converted to a 3-slice (red, green, blue) stack and "thresholded" to highlight only IHC positive cells (Suppl. Fig. 1B). Outlines of particles on the "thresholded" image were then overlain on the original image to ensure accurate coverage of positively labeled cells (Suppl. Fig. 1C). The area of these particles was then measured and the particle area was divided by the total cellular area in that image and multiplied by 100 to create the %CD204+ value. The evaluator was blinded to patient outcomes during image capture and IHC quantification. The intensity of CD3, FOXP3 or CD204 staining was not evaluated.

The average quantity of CD3 or FOXP3 staining (cells/mm^2), and %CD204+ was then determined across all 3 cellular fields evaluated for each sample. In cases where one of the cellular fields was not evaluable due to disruption of the tissue on the slide, the quantity of infiltration in the remaining 2 fields was averaged. The percentage of FOXP3+ cells of CD3+ cells (FOXP3/CD3%) was calculated by dividing the average number of FOXP3+ infiltrates per mm^2 across the 3 cellular areas, by the average number of CD3+ infiltrates per mm^2 across the 3 cellular areas and multiplying by 100.

2.5 Endpoints and statistical analysis

Disease free interval (DFI) was defined as time from amputation to documented or suspected metastasis or to death from any cause. DFI was censored for dogs alive without evidence of metastasis and dogs without evidence of metastasis when lost to follow-up. Overall survival time (ST) was defined as time from amputation to death from any cause and was censored for dogs alive at study's end or lost to follow-up.

Clinical and pathological factors evaluated for association with DFI and ST included tumor location (proximal humerus vs other), histologic subtype (osteoblastic vs other), pre-surgical ALP, mitoses per 3 hpf, and chemotherapy treatment interval (3 weeks vs 4 weeks). Immune cell infiltrates were also evaluated for their association with DFI and ST, including CD3 (positive cells/mm^2), FOXP3+ infiltrates (positive cells/mm^2), FOXP3/CD3%, and %CD204+. "High" and "low" infiltrates were determined by the median, and the top quartile vs. the bottom 3 quartiles, in 2 separate analyses. Clinical and pathological factors including age, pathological fracture, decalcification of the tissue, tumor location (proximal humerus vs. other), histologic subtype (osteoblastic vs. other), mitoses per 3 hpf, and pre-surgical ALP, were evaluated for their association with immune cell infiltration.

The Kaplan-Meier method was used to estimate median DFI and ST (MST), as well as the 2-yr disease-free and survival rates. The log-rank test was used to compare DFI and ST between groups. Any relationships between continuous variables (such as immune cell infiltrates and age) were evaluated using Spearman's correlation coefficient, and associations between categorical variables and immune infiltrates were determined using a Mann-

Whitney test. Statistical analyses were performed using commercial software (GraphPad Prism version 7.0a, San Diego, CA) and 2-sided p-values < 0.05 were considered significant.

3. Results

3.1 Clinical characteristics

Thirty dogs were enrolled between January 2010 and January 2014. The mean age at amputation was 9.1 years (median = 8.9 years; range = 2.5–15 years). There were 15 castrated males, 13 spayed females, and 2 intact males. There were 8 mixed breed dogs, 6 Labrador retrievers, and 2 or fewer dogs of 12 other breeds. Nineteen dogs had normal serum alkaline phosphatase (ALP) levels prior to amputation, 10 had high ALP levels, and results were not available for one dog. Tumors were located in the distal radius (10 dogs), distal tibia (6 dogs), proximal humerus (5 dogs), distal femur (4 dogs), proximal tibia (4 dogs), or proximal ulna (1 dog). Nineteen tumors were classified by the pathologist as osteoblastic subtype, 3 were chondroblastic, 3 were fibroblastic, 3 were mixed type, and one each was giant cell type or anaplastic. The median number of mitotic figures per 3 high power fields was 3.3 (range, 1–10.2). The median time from amputation to chemotherapy initiation was 17 days (range = 10–29 days). Twenty-three dogs (77%) completed their prescribed 6-dose treatment protocol. Six dogs stopped treatment early due to progressive disease after 5 doses (3 dogs), 4 doses (1 dog), or 2 doses (2 dogs). One dog stopped treatment after 4 doses at the owner's discretion. The dose of carboplatin was reduced to 255–270 mg/m^2 due to toxicity in 3 dogs. Treatment delay to a 28-day cycle after carboplatin was required in 12 dogs (40%) due to a late neutrophil nadir.

Eight dogs did not complete the recommended follow-up schedule after completion of chemotherapy. These dogs were evaluated at the VMTH for a median of 208.5 days (range = 128–687 days) and data collection beyond this point occurred through communication with referring veterinarians and owners. DFI was censored at 714 days for 1 dog that was lost to follow-up without metastasis. At study's end, 26 dogs had died. Four dogs underwent necropsy and metastatic OSA was diagnosed in all dogs. ST was censored for 4 dogs including two dogs that were alive at 1687 and 1924 days and 2 dogs that were lost to follow up at 642 and 714 days. The median DFI for all dogs was 392 days and the MST was 455 days (Fig. 1A-B). Two years after diagnosis, 20.0% (95% CI = 8.1% - 35.6%) of dogs were disease-free and 25.9% (95% CI = 11.9% - 42.5%) of dogs were alive. None of the clinical or pathological factors evaluated were significantly associated with DFI or ST in this population (Table 1).

3.2 Quantification of infiltrating immune cells

Of the 30 dogs included in survival analyses, FFPE tissue blocks were available in 26 dogs. Of these, tissue samples from 2 of these dogs were considered too necrotic and poorly cellular to evaluate immune cell infiltrates. Sections from one other tissue block consistently sloughed off the slide during processing for CD3 IHC, resulting in 23 dogs evaluable for CD3+ cellular infiltrates and 24 dogs evaluable for FOXP3+ and CD204+ cellular infiltrates. CD3+ and FOXP3+ infiltrates were quantified by counting the number of positive cells within a cellular area (positive cells/mm^2), and CD204+ infiltrates were calculated as a

percentage area of the cellular area (%). Staining was cytoplasmic for CD3 and CD204, and nuclear for FOXP3, as expected for these markers.[48,52,53] Representative images of tumors with infiltrates above and below the median are shown in Figure 2A-C. CD3+ infiltrates were present in every tumor, unlike FOXP3+ infiltrates, which were absent from 4 tumors. The median number of CD3+ cells was 45.8 /mm^2 (range = 4.6 – 607.6 cells/mm^2), the median number of FOXP3+ cells was 8.5 /mm^2 (range = 0 – 163.1 cells/mm^2), the median FOXP3/CD3% was 16.8% (range = 0 – 134.8%), and the median %CD204+ was 4.7% (range = 1.3% - 23.3%) of the cellular areas. There was no significant difference in immune cell infiltrates recorded between dogs with or without a pathological fracture at the time of amputation, or between samples that had undergone decalcification or not (data not shown). Interestingly, all 3 immune cell infiltrates correlated well with each other (Fig. 3A-C).

3.3 Association of immune cell infiltrates with clinical characteristics

The association of immune cell infiltrates with outcome was then evaluated (Table 2). Dogs were initially differentiated as having “high” or “low” infiltrate levels, by the median of each infiltrate. Only high %CD204+ infiltration was associated with improved DFI (P = 0.016; Fig. 4A). However, high %CD204+ infiltration was not prognostic for ST (P = 0.202; Fig. 4B). There was a numerical, but statistically insignificant, increase in median DFI and MST for dogs with high CD3+ (P = 0.484, P = 0.937; Suppl. Fig. 2A-B), and high FOXP3+ (P = 0.224, P = 0.287; Suppl. Fig. 2C-D) infiltration, and for dogs with a high FOXP3/CD3% (P = 0.246, P = 0.276; Suppl. Fig. 2E-F).

Next, we evaluated whether the dogs with the highest amount of inflammatory infiltrates experienced a different outcome to the remaining dogs (Table 3). Here dogs were defined as having “high” infiltration if the quantity of inflammatory infiltrates was within the top quartile, or “low” infiltration if they fell within the bottom 3 quartiles. Once again, high CD204+ infiltration was associated with improved DFI (P = 0.043; Suppl. Fig. 3A), while this parameter did not have a significant effect on ST as an endpoint (P = 0.201; Suppl. Fig. 3B). Furthermore, when high and low values were separated by the top quartile, as opposed to the median, a high FOXP3/CD3% was associated with significantly improved DFI and ST (P = 0.016 and P = 0.014, respectively; Suppl. Fig. 3C-D). No significant difference in DFI or ST was again detected between high and low CD3 (P = 0.528, P = 0.885; Suppl. Fig. 3E-F) or FOXP3 (P = 0.312, P = 0.600; Suppl. Fig. 3G-H) infiltration.

Finally, we determined if any clinical or pathologic factors could be associated with immune cell infiltration. A summary of these findings is displayed in Table 4. Interestingly, we observed a significant decrease in CD204+ infiltrates in tumors located in the proximal humerus, compared to all other locations (P = 0.016; Fig. 5A). A trend towards decreased CD3+ and FOXP3+ infiltrates, and FOXP3/CD3%, in proximal humeral lesions was also detected (P = 0.067, P = 0.090, and P = 0.231, respectively; Fig. 5B-D). No significant difference in immune cell infiltration was detected in dogs delineated by the remaining categorical variables (Table 4). No correlation with age at amputation was observed (CD3: P = 0.486; FOXP3: P = 0.495; FOXP3/CD3: P = 0.645; CD204: P = 0.563).

4. Discussion

The immune microenvironment within tumors is thought to play a critical role in determining cancer behavior. Furthermore, understanding the characteristics of host-tumor interactions can uncover immunotherapeutic targets, as well as predictive and prognostic biomarkers. In order to provide baseline information regarding the immune response to cOSA, we quantified lymphocytic and macrophage infiltrates in the primary tumors of dogs treated with amputation and 6 doses of adjuvant carboplatin. We found CD204+ macrophages to be particularly abundant, however a great deal of variation was evident for all 3 immune markers evaluated including CD3, FOXP3, and CD204. Furthermore, greater intratumoral infiltration with CD204+ macrophages was associated with a prolonged DFI. Together these findings suggest cOSA is an immunogenic tumor, with tumor infiltrating macrophages potentially playing an inhibitory role in the progression of metastatic disease, similar to findings in human studies.[27,29]

The outcomes of the 30 dogs with cOSA treated with amputation and 6 doses of adjuvant carboplatin chemotherapy reported herein are within the ranges reported previously for amputation and adjuvant carboplatin chemotherapy, indicating that this cohort is likely a fair representation of cOSA patients. In fact, outcomes are quite similar to those from a previous prospective study in which 25 dogs received 6 doses of adjuvant carboplatin.[16] The median DFI in that study was 425 days compared to 392 days here and the MST was 479 days compared to 455 days here. These results compare favorably with previously published retrospective studies reporting outcome in dogs prescribed 4 or 6 doses of adjuvant carboplatin.[2,8] One retrospective study found a significantly lower risk of metastasis and death in dogs prescribed 6 doses of carboplatin in univariate analysis compared to other chemotherapy protocols, but that study did not identify significant differences in outcome in multivariate analysis.[2] Given these differences between the findings of prospective and retrospective reports on dogs treated with 6 doses of carboplatin, it is possible that confounding factors inherent to retrospective studies may have led to an underestimation of survival times.[2,8] In an attempt to maximize the strength of the available prospective clinical information, we reported the outcome of all 30 dogs that were prospectively evaluated, despite the availability of tissue samples in only 26.

Consistent with findings in hOSA, elevated tumor infiltrating macrophages above the median in cOSA were associated with an improved DFI in this study.[27,29] In addition, dogs with tumor infiltrating macrophages in the top quartile experienced a significantly prolonged DFI compared to those with values in the bottom 3 quartiles, suggesting a “dose-response” correlation might exist in a larger cohort of dogs. Macrophage infiltration of tumors is thought to be mediated predominantly by the production of monocyte-attracting chemokines such as CCL2 (monocyte chemoattractant protein-1) and CCL5 that can be produced by tumor cells, fibroblasts, endothelial cells, and macrophages within the tumor miroenvironment.[54] Interestingly, compared to healthy dogs, those with cOSA have been found to express significantly less chemokine receptors such as CCR2 on their circulating monocytes[32]. CCR2 facilitates cell migration towards CCL2, and therefore this study concluded that systemic aberrations to the migratory ability of monocytes may facilitate their peripheral sequestration and promote tumor progression in dogs with cOSA.[32] Tumor-

infiltrating macrophages however, are often described as becoming skewed towards the more "tumor-promoting" M2 (alternative) phenotype due to exposure to T helper type 2 (Th2) cytokines such as IL-4 and IL-13, and the immunosuppressive cytokines IL-10 and transforming growth factor-β (TGF-β).[55,56] In contrast, macrophages stimulated by microbial products such as lipopolysaccharide, with or without concurrent exposure to proinflammatory cytokines such as tumor necrosis factor α (TNFα) and interferon γ (IFNγ) can become "anti-tumor" M1 (classical) phenotype macrophages.[55,56] However, this paradigm does not allow for the broad array of functional and phenotypic characteristics of macrophages particular to certain tumor types.[56,57] Notwithstanding the diversity within macrophage subsets, reports on several human cancer types as well as canine mammary carcinomas, show macrophage infiltration as correlating with a worse prognosis.[57–60] In contrast however, most studies in hOSA have reported tumor infiltrating macrophages as being associated with improved outcomes,[27–29] although some conflicting data exist.[43] Furthermore, most studies in hOSA have shown total (CD14+[27] or CD68+[28,29]) macrophage infiltrates, or even M2 phenotypes (CD163+[29]) to positively correlate with outcome, indicating that differential macrophage polarization is likely not the cause of this prognostic finding in hOSA. Indeed, we identified macrophages using CD204 in our study due its well-documented cross-reactivity with canine tissue, but this marker is also commonly associated with M2-type macrophages.[61,62] Supporting the notion of macrophages playing a potential antitumor role in OSA, are the findings of clinical efficacy elicited by macrophage-activating therapeutics such as L-MTP-PE, and the recent observation that tumor-infiltrating macrophages play a critical role in mediating response to PD-1/PDL-1 inhibition in murine models of OSA metastases.[20,21,63,64] These studies and others describe the mechanistic basis for the efficacy of these agents to be the activation of M1-type macrophages and their induced expression of soluble factors that cause direct inhibition of OSA cells.[64–66] Taken together, our findings are in agreement with previous studies in humans that suggest macrophages may have a tumor-suppressive role in OSA. Furthermore, these data provide supporting evidence for the role of naturally occurring cOSA in modeling the tumor immune microenvironment in hOSA.

While dogs with cOSA of their proximal humerus did not experience a significantly decreased DFI or ST in our study, our finding of a significant association between lower CD204+ macrophage infiltrates and a proximal humeral cOSA location indicate that macrophage infiltrates are likely not an independent prognostic factor. Indeed, a meta-analysis including 55 papers identified the proximal humeral location as one of only 2 prognostic factors to be significantly associated with a worse prognosis in dogs without gross metastatic disease at the time of diagnosis.[67] These findings do however raise the possibility that differences in the immune microenvironment between proximal humeral cOSA and other primary appendicular bone locations could mediate this known difference in associated clinical outcomes. Despite the prognostic significance of proximal appendicular locations in humans,[68] potential correlations of immune infiltrates with the primary tumor site are rarely reported in hOSA.[28,29,43,50,69] Buddingh et al. however observed CD14+ infiltrates to be an independent positive prognostic factor when humeral location was included in the model, suggesting tumor site and monocyte/macrophage infiltration were not associated in this study.[27] In fact, to the authors' knowledge no study has shown consistent

differences in immune infiltrates between tumors located in different bones, although growing evidence suggests the tissue-dependent recruitment of certain types of macrophages across soft tissue types.[70–72] Alternatively, the smaller number of dogs with proximal humeral lesions in this study raises the possibility of their association with lower CD204+ infiltrates as being due to type I error. Further evaluation of this finding in a larger cohort of dogs is therefore warranted in the future.

Immune cell infiltrates in cOSA were positively correlated with each other in this cohort. Similar to past reports in hOSA, tumors with high lymphocyte infiltrates typically also had high macrophage infiltrates.[28,29] These findings agree with our understanding of tumor-infiltrating macrophages as the primary producers of lymphocyte recruiting chemokines such as CCL5, CXCL9, and CXCL10.[73,74] While our results may be reflective of cOSA recruiting fairly consistent proportions of leukocyte types across individuals, it is also possible that a more detailed evaluation of leukocyte subsets will elucidate more variability in the future. Nevertheless, despite the positive correlation between CD204+ macrophages and lymphocytes in addition to the positive effect of CD204+ macrophages on outcome in this study, CD3+ infiltrates were not associated with a better prognosis. This may be predominantly explained by the lack of lymphocyte subset markers used in this study, other than FOXP3, since we were unfortunately limited by the availability of only FFPE tissue, and the cross-reactive or anti-canine antibodies that bind such tissues. For example, in hOSA high CD8+ infiltrates have been correlated with a survival benefit, but this marker requires fresh frozen tissue for evaluation in dogs.[29,75] Furthermore, known correlations between lymphocyte phenotypes and specific functions have been used to elucidate other prognostically significant lymphocyte subsets such as Th1, Th2, Th17, γ/δ-T cells, CD8+CD103+T cells, and lymphocyte memory subsets, in addition to prognostic markers of T cell exhaustion, in a variety of human tumors.[39,76–82] Indeed, the variability of CD3+ infiltrates in predicting prognosis in certain canine tumor types may be explained by the heterogeneity of TILs function that exists, emphasizing the need for more detailed phenotypic analyses of lymphocyte subsets in future studies.[83–85]

Interestingly, when evaluating the DFI and ST of dogs with immune cell infiltrates in the top quartile, compared to those in the bottom 3 quartiles, we also identified a high proportion of infiltrating FOXP3+ cells relative to CD3+ cells (FOXP3/CD3%), to be positively associated with outcome. This result is somewhat confounding due to a previous report by Biller et al., which identified high FOXP3+ T cells relative to CD8+ T cells in the blood as being associated with a negative prognosis.[25] However, as previously discussed we were unable to stain for CD8+ cells in this study, making a direct comparison to the study by Biller et al. impossible. Furthermore, it is feasible that discordance may exist between immune cell populations found in the intratumoral microenvironment and those found in the peripheral blood of dogs with cOSA, similar to what has been reported for various immune subsets in other tumor types.[35–38] Several studies in both dogs and humans have however reported the presence of FOXP3+ cells within tumors to be a negative prognostic factor owing to the fact that these cells are generally thought to have regulatory T cell (Treg) activity.[86–90] More recently however, human studies have observed FOXP3 to be expressed by some non-lymphoid normal and cancerous cells, in addition to being transiently expressed by non-regulatory T cells during their activation.[91–93] Indeed, this likely explains the variability of

FOXP3-expressing cells as being associated with a poor, neutral, or good prognosis in human cancers.[90] The broad functionality of FOXP3+ cells within tumors has unsurprisingly led to their prognostic significance being determined by their identification using multiple lymphoid markers, as well as the tumor type being studied.[90] In summary, our finding of high FOXP3/CD3% being associated with an improved prognosis could either be reflective of the anti-metastatic activity of FOXP3+ cells in the cOSA context, or secondary to type I error as a result of the lower number of dogs in the top quartile or our methods of single-staining adjacent sections of tissue rather than double-staining.[90]

Despite the prospective nature of this study, there were several limitations that may have affected our outcome analyses. Firstly, 8 dogs did not complete the recommended follow-up schedule and this could have resulted in overestimation of DFI in some cases. To minimize this effect, follow-up data from dogs that missed appointments were collected in real time during the study period through contact with referring veterinarians and pet owners. Nonetheless, 2 dogs were lost to follow-up and repeated attempts to contact the owners failed. Additionally, some dogs did not have a complete diagnostic work-up at or around the time of death, and only 4 of the 26 dogs that had died underwent necropsy to definitively determine cause of death. In order to minimize the effects of unknown cause of death on outcome variables, metastasis was assumed if cause of death was unknown and death from any cause was used in survival calculations. Despite this, it is possible that data loss due to incomplete follow-up may have influenced study results.

In conclusion, these results suggest that dogs with appendicular osteosarcoma prescribed 6 doses of carboplatin chemotherapy may have a modest DFI and ST benefit when compared to previously published data from dogs prescribed other chemotherapy protocols. These findings may be due, in part, to a higher chemotherapy completion rate than has previously been reported with this chemotherapy protocol. Within this cohort of dogs, we identified CD204+ infiltration above the median to be associated with an improved DFI and appendicular tumor locations other than the proximal humerus. Further studies are needed to evaluate the possibility that poor macrophage recruitment is a contributing factor to the rapid progression of cOSA arising from the proximal humerus. Taken together, these data support macrophage recruitment and activation as immunotherapeutic strategies for treating cOSA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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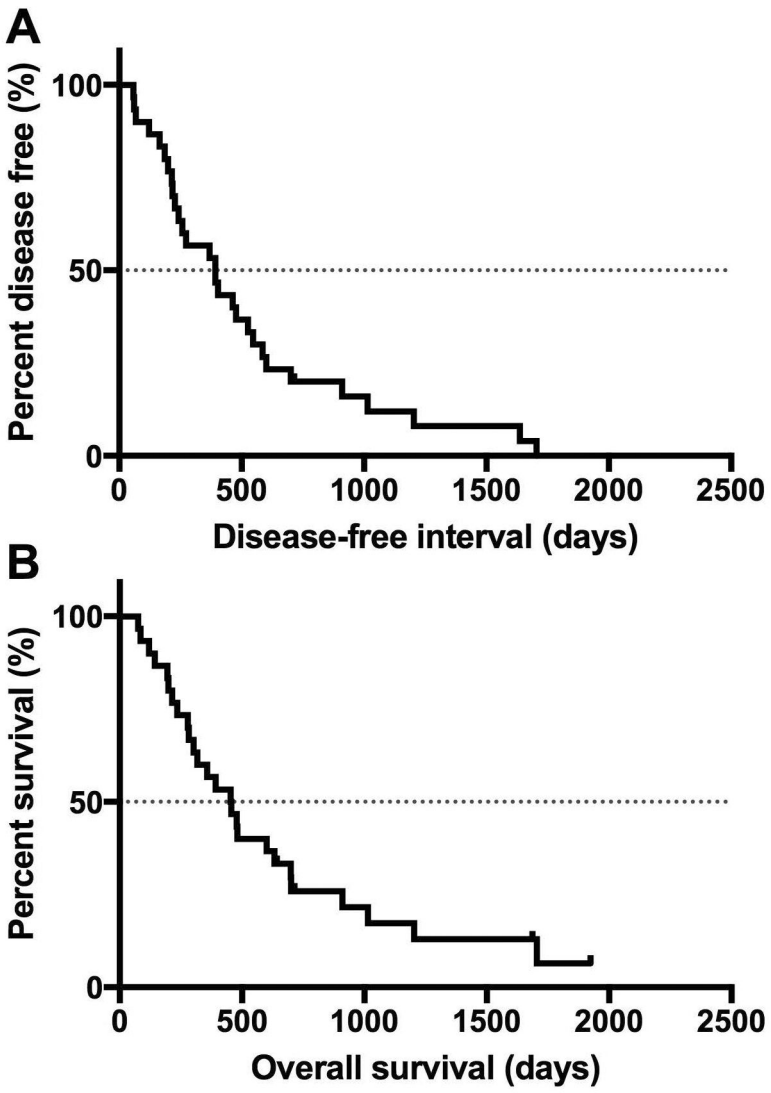

Figure 1:

Outcome of all 30 dogs with cOSA treated prospectively with amputation and 6 doses of adjuvant carboplatin chemotherapy. Kaplan Meier curves summarizing the A) DFI and B) survival times are shown. Tick marks represent censored events, and the horizontal dotted line indicates the 50% disease-free or survival threshold.

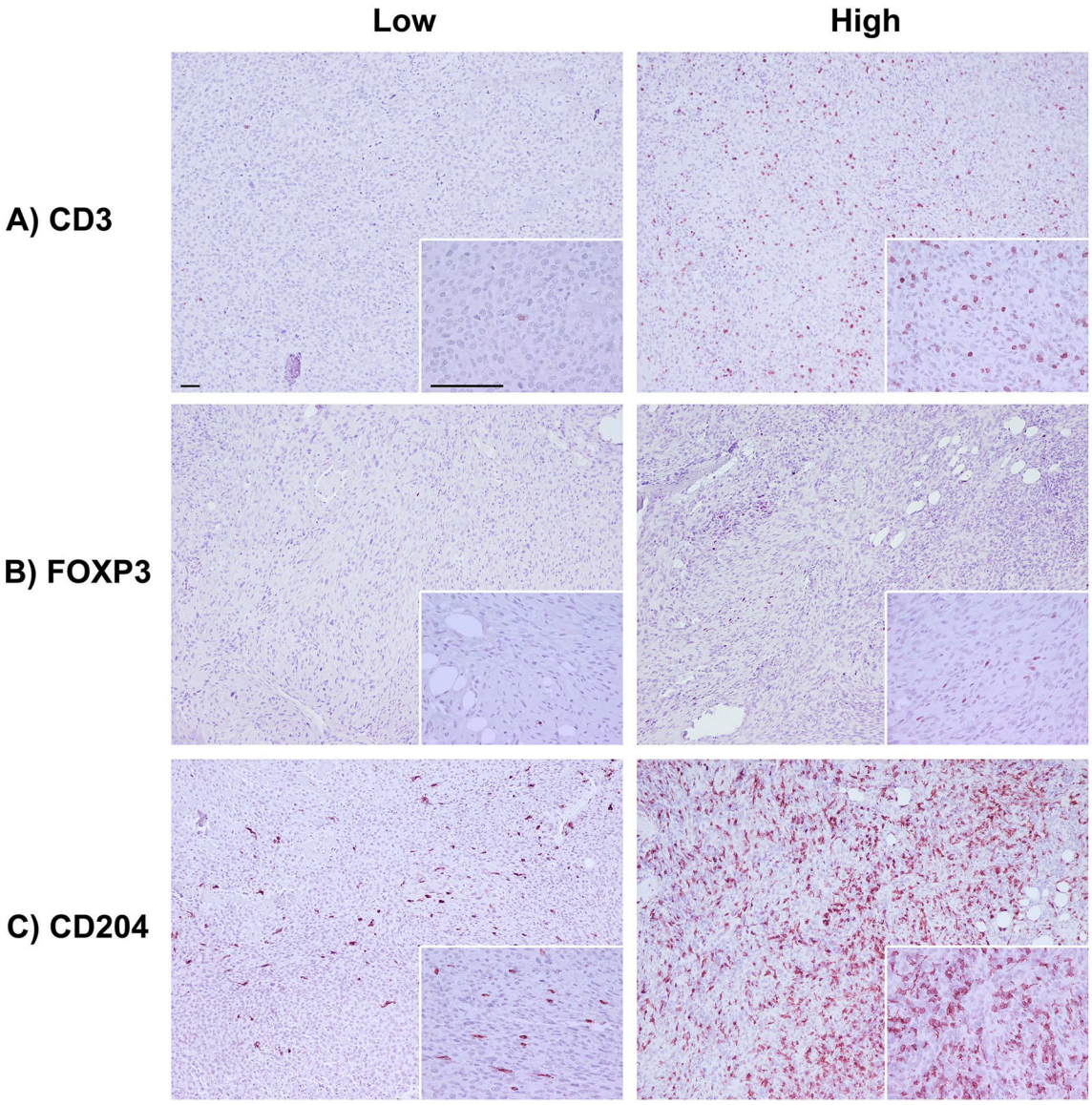

Figure 2:

Representative examples of IHC stained cOSA sections for immunological targets. Low (below median) and high (above median) infiltrated cOSA tissue samples are shown. A) CD3, B) FOXP3, and C) CD204 expressing infiltrates. 100× magnification with 400× magnification insets. Bar represents 50um length.

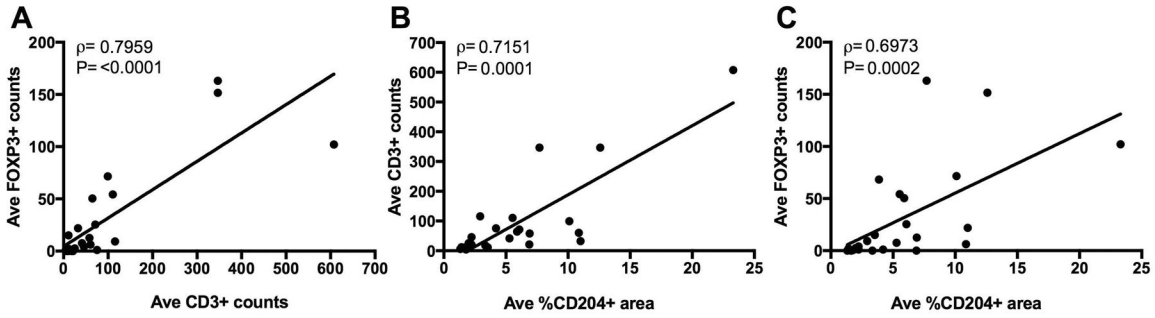

Figure 3:

Correlations between various inflammatory infiltrates in cOSA. A) FOXP3+ vs. CD3+ cells/mm^2, B) CD3+cells/mm^2 counts vs. %CD204+, and C) FOXP3+ cells/mm^2 vs. %CD204+, within each tumor. A line of best fit has been plotted, and Spearman's correlation coefficient (ρ) and P values are shown on each graph.

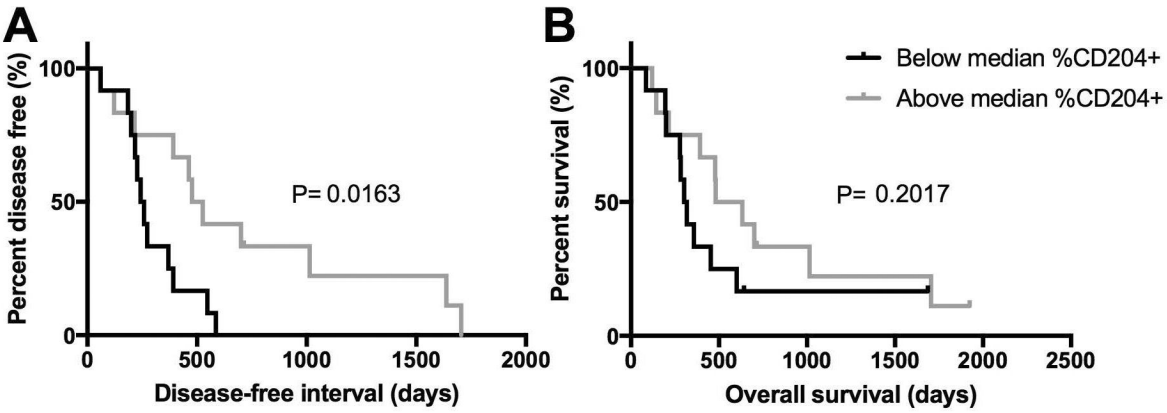

Figure 4:

Association of macrophage tumor infiltration with outcome in cOSA. Kaplan Meier curves summarizing the A) DFI, and B) survival times of dogs with %CD204+ values above (grey line) or below (black line) the median. Tick marks represent censored events. P values are shown.

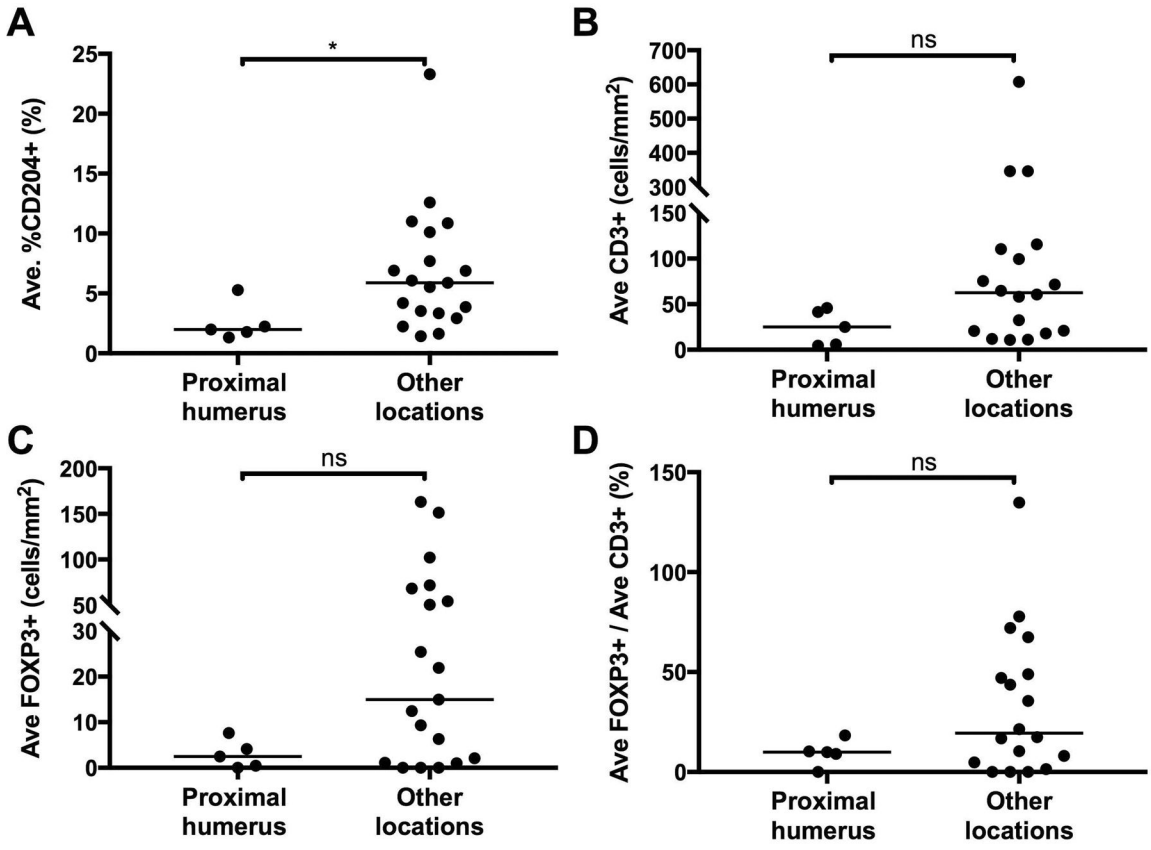

Figure 5:

Immune infiltrates in proximal humeral cOSA vs. other appendicular bone locations. A) %CD204+, B) CD3+ cells/mm^2, C) FOXP3+ cells/mm^2, and D) FOXP3/CD3% within primary tumors originating from the humerus or other locations. Each dot represents the average value for that tumor across 3 images. Overlain horizontal lines represent the median. * = P<0.05; ns = not significant.

Table 1-

Association of clinical and pathological factors with DFI and ST

		Median DFI (days)	*P value*	MST (days)	*P value*
Tumor location	Proximal humerus	243	*0.144*	318	*0.352*
	Other	404		479	
Histologic subtype	Osteoblastic	313.5	*0.901*	406.5	*0.279*
	Other	434.5		467.5	
Pre-surgical ALP	Normal	404	*0.706*	479	*0.373*
	High	236.5		277	
Mitoses per 3 hpf	≤5	321	*0.530*	405.5	*0.661*
	>5	433.5		467.5	
Chemotherapy treatment interval	3 weeks	235	*0.213*	310	*0.695*
	4 weeks	433.5		480	

DFI= disease-free interval; MST = median overall survival time; ALP= alkaline phosphatase; hpf= high power field.

Table 2-

Association of immune cell infiltration (below or above median) with outcome

	Below/above median	Median DFI (days)	2-yr disease-free rate	P value	MST (days)	2-yr survival rate	P value
CD3	Below	251	16.7%	*0.484*	338	33.3%	*0.937*
	Above	463	18.2%		479	18.2%	
FOXP3	Below	251	8.3%	*0.224*	310	12.5%	*0.287*
	Above	470	25%		480	33.3%	
FOXP3/CD3	Below	251	8.3%	*0.246*	310	12.5%	*0.276*
	Above	477	27.3%		481	36.4%	
CD204	Below	251	0%	***0.016***	310	16.7%	*0.202*
	Above	502	33.3%		557	33.3%	

DFI= disease-free interval; MST = median overall survival time

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Table 3-

Association of immune cell infiltration (above or below top quartile) with outcome

	Below/above top quartile	Median DFI (days)	2-yr disease-free rate	P value	MST (days)	2-yr survival rate	P value
CD3	Below	369	11.8%	*0.528*	479	22.1%	*0.885*
	Above	304	33.3%		304	33.3%	
FOXP3	Below	321	20.8%	*0.312*	418	11.1%	*0.600*
	Above	392	33.3%		423	33.3%	
FOXP3/CD3	Below	243	5.9%	***0.016***	302	8.8%	***0.014***
	Above	781	50%		1360	66.7%	
CD204	Below	321	5.6%	***0.043***	375	50%	*0.201*
	Above	857	50%		857	16.7%	

DFI= disease-free interval; MST = median overall survival time

Table 4-

Association of clinical and pathological factors with immune cell infiltration

		Median CD3+ (cell/mm^2)	P value	Median FOXP3+ (cell/mm^2)	P value	Median FOXP3/CD3 (%)	P value	Median CD204 (%)	P value
Tumor location	Proximal humerus	25.2	*0.067*	2.5	*0.090*	9.9	*0.231*	2.0	***0.016***
	Other	62.7		15.0		19.4		5.9	
Histologic subtype	Osteoblastic	52	*0.566*	7.0	*0.897*	13.6	*0.660*	6.1	*0.468*
	Other	32.5		18.4		35.6		4.0	
Pre-surgical ALP	Normal	41.6	*0.783*	10.1	*0.855*	18.4	*0.521*	4.7	*0.758*
	High	99.5		9.3		10.3		3.3	
Mitosis per 3 hpf	≤5	41.6	*0.548*	7.0	*0.800*	16.8	*0.693*	4.0	*>0.999*
	>5	61.5		10.9		15.9		5.7	

ALP= alkaline phosphatase; hpf= high power field.