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Obesity Does Not Exacerbate the Protumorigenic Systemic Environment in Sarcoma Subjects

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Abstract

Sarcomas are a rare but fatal tumor type that accounts for <1% of adult solid malignancies and ~15% of childhood malignancies. Although the use of immunotherapy is being actively investigated for other solid tumors, advances in immunotherapy for sarcoma patients are lacking. To better understand the systemic immune environment in sarcoma patients, we performed a detailed multiplex analysis of serum cytokines, chemokines, and protumorigenic factors from treatment-naive subjects with localized, high-grade sarcoma. Because obesity is a major healthcare issue in the United States, we additionally examined the effects of obesity on serum protein profiles in our sarcoma subject cohort. We found that the systemic host environment is profoundly altered to favor tumor progression, with epidermal growth factor, angiopoietin-2, vascular endothelial growth factor A, IL-6, IL-8, and MIP-1β all increased relative to tumor-free controls (all $p < 0.05$). Surprisingly, we found that obesity did not exacerbate this protumorigenic profile, as epidermal growth factor and IL-8 decreased with increasing subject body mass index (both $p < 0.05$ versus normal or overweight subjects). The Th2-related cytokines IL-4, IL-5, and IL-13 were also decreased in the presence of obesity. Thus, although the systemic environment in sarcoma subjects favors tumor progression, obesity does not further aggravate the production of protumorigenic factors.

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DISCLOSURES

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INTRODUCTION

Sarcomas are a rare, but extremely fatal, tumor type that accounts for <1% of adult solid malignancies and ~15% of childhood malignancies in the United States. Although more than 50 histologically distinct subtypes exist, sarcomas are generally separated into two main groups: bone and soft-tissue tumors, with the latter comprising the majority of adult patient cases (1). The etiology of sarcoma is not well understood; nevertheless, a number of risk factors have been identified that include exposure to ionizing radiation, carcinogens, and viral infections (2).

The general approach to treatment of sarcoma patients has remained unchanged for decades. Surgery remains the primary standard treatment for soft-tissue sarcoma, with radiotherapy a common adjuvant to decrease local recurrence and allow for function-preserving surgery. However, in patients who present with or develop distant metastases, options for systemic treatment are limited and of marginal benefit. With five-year survival rates dropping from 90% for localized soft-tissue sarcomas to 10–20% for metastasized sarcomas (3), it is clear that new therapeutic options for metastasized sarcomas are needed. The human immune system serves as a critical line of defense against cancer development. In 1891, William Coley demonstrated one of the first successful examples of immunotherapy by injecting sarcoma patients with streptococcal organisms (4). Despite this breakthrough nearly 120 y ago, immunotherapy research in sarcoma now lags behind other solid tumor types, as noted by recent advancements in the treatment of cancers such as melanoma, renal cell carcinoma, and non-small cell lung cancer (5). With the success of immunotherapeutic intervention in these tumor types, there has been a renewed interest in immunotherapy for sarcoma patients.

Currently, it is estimated that 35% of the adult population in the United States is categorized as obese (body mass index [BMI] ≥30 kg/m^2), and this percentage is expected to exceed 40% by 2030 (6). Several health complications have been associated with obesity, including increased risks for cardiovascular disease and cancer development. These facts have generated interest in examining potential associations between obesity and sarcoma risk and outcomes. Early case studies found that the risk of developing soft-tissue sarcoma rose with increased body weight (7, 8). A more recent study observed that obesity did not serve as an independent risk factor in affecting survival outcomes and additionally did not appear to affect local recurrence or wound complication rates for patients with soft-tissue sarcomas (9). This was contrasted by a similar study identifying obesity as an independent predictor of wound complications in soft-tissue sarcoma resection (10). Furthermore, it was determined that a high BMI at diagnosis was associated with a reduced overall survival in pediatric osteosarcoma subjects (11). From these studies, it is clear that the associations between obesity and sarcoma must be investigated further. This will be particularly important in the context of cancer immunotherapy, as obesity is known to be associated with long-term systemic inflammation, which has been linked to cancer progression, immunomodulation, and immunotherapeutic failure in multiple preclinical murine tumor models (12–16).

Previous studies have examined the links between the immune system and the development and progression of sarcoma. However, to the best of our knowledge, no study has yet to

compile an in-depth profile of the immune response to human sarcoma to identify systemic inflammatory changes that could be further exploited in immunotherapy. Furthermore, no study has yet examined the role of obesity on the inflammatory or protumorigenic milieu in sarcoma patients. To this end, we sought to accomplish two goals: 1) to provide a comprehensive baseline cytokine and chemokine profile in treatment-naive human sarcoma patients, and 2) to determine the extent to which obesity contributes to a protumorigenic systemic environment in sarcoma patients. This study should serve as a springboard for additional, and much needed, advances in immunotherapeutic design and development for sarcoma patients.

MATERIALS AND METHODS

Biospecimen collection

Banked serum from human sarcoma subjects was obtained from the University of Iowa's Melanoma and Sarcoma Tissue Bank (MAST) with Institutional Review Board approval 201512776. All biospecimens tested were from treatment-naive subjects with a confirmed diagnosis of high-grade (>5 cm diameter tumor) sarcoma not metastatic at the time of enrollment. Exclusion criteria included prior history of sarcoma and/or any additional tumor types or prior cancer treatment. Serum from tumor-free donors was collected following informed consent as per the University of Iowa Institutional Review Board 201209718, and frozen at −80°C until use.

Human cancer and human cytokine panels

Human cancer biomarker analysis was accomplished with the Bio-Rad Bio-Plex Pro Human Cancer Biomarker Panel 2, 18-plex according to the manufacturer's instructions (catalog number 171AC600M; Bio-Rad Laboratories). Human cytokine analysis was accomplished with the Bio-Rad Bio-Plex Pro Human Cytokine 17-plex assay according to the manufacturer's instructions (catalog number M5000031YV; Bio-Rad Laboratories).

Statistical analyses

Sarcoma subject serum was randomized to each plate to ensure there was no inherent bias from different plate runs, and all samples were run in duplicate. A total of 50 beads was collected for each event. Data obtained from Luminex-based assays were carefully examined to ensure the most accurate representation and conclusions could be drawn from these data. The standard curves for each individual analyte examined were cross-checked against the same curves on all other plates to evaluate similarity in controls across plates. Individual analytes from the panels were checked for analyte call values; only those analytes that had a >80% value call (meaning the analysis software was able to determine a numerical concentration for >80% of the subject samples tested for that analyte, rather than giving an out of range error that was too low) were included in the analyses. To highlight those analytes that had a <80% value call, we grouped and discussed these separately, and data are presented in Supplemental Fig. 1. For example, in instances where analytes were categorized by BMI status, if one of the categories had a <80% value call, the data for that analyte are shown in Supplemental Fig. 1. For statistical analyses, concentrations that were lower than

the detectable threshold (out of range <) were substituted as the lowest possible numerical value for that analyte.

Statistical analyses were performed using Prism, Version 6.07 (GraphPad). Gaussian distribution was assessed using the D'Agostino–Pearson omnibus normality test ($p > 0.05$ threshold). Data were analyzed either with an unpaired *t* test with (as denoted by carets; ^) or without (as denoted by asterisks; *) Welch correction (as needed), or by using an ordinary one-way ANOVA with post hoc multiple comparisons analyzed using Tukey multiple comparisons test (as denoted by asterisks; *; comparisons of three). Nonparametric analyses were performed either with an unpaired Mann–Whitney *U* test (as denoted by number/pound signs; #), or by using a one-way Kruskal–Wallis test with post hoc multiple comparisons analyzed using Dunn multiple comparisons test (as denoted by number/pound signs; #; comparisons of three). Calculated *p* values for each analyte are indicated in each figure: *,#,^$p < 0.05$, **,##,^^$p < 0.01$. Statistical significance of linear regressions is listed within the figure.

RESULTS

Characterization of subject cohort

We began our study by obtaining serum from sarcoma subjects through the MAST bank. A total of 50 sarcoma subjects met our inclusion or exclusion criteria as detailed in the *Materials and Methods*, and serum samples from these individuals were retrieved and analyzed. In our cohort, sarcoma subjects had an average age of 58 y, and the cohort composition was 52% female and 48% male. Due to the nature of sarcoma subtyping, we had multiple different types of sarcoma (osteosarcoma, liposarcoma, leiomyosarcoma, etc.) represented, as detailed in Table I. We additionally collected serum samples from healthy donor controls. Healthy control subjects had an average age of 54 y, and were 51% female and 49% male. All sarcoma subjects were treatment naive, allowing us to evaluate alterations in analyte levels prior to intervention by standard therapy. We subsequently performed Luminex-based assays for both human cancer biomarker and human cytokine and chemokine analysis on all collected serum samples.

Subjects with sarcoma exhibit increases in multiple protumorigenic and proinflammatory cytokines

As detailed in Fig. 1, 10 of 21 proteins examined were significantly elevated in serum from sarcoma subjects as compared with healthy donor controls. In addition, five proteins were significantly decreased in sarcoma subjects, whereas six proteins were not significantly altered by the presence of a tumor. As angiogenesis, the process by which new blood vessels are formed, is a hallmark of cancer (17), we examined multiple angiogenic factors. When we examined the vascular endothelial growth factor (VEGF) family, we found that systemic levels of several VEGF proteins were altered in sarcoma subjects. VEGF-A was increased compared with healthy controls ($p = 0.0003$), whereas placental growth factor and VEGF-D were decreased ($p = 0.002$ and <0.0001, respectively). VEGF-C was equivalent in healthy controls and sarcoma subjects. Angiopoeitin-2, another protein involved in angiogenesis, was also increased in sarcoma subjects relative to controls ($p = <0.0001$). We additionally

investigated systemic concentrations of proteins involved in cellular proliferation. The epidermal growth factor (EGF) receptor ligands EGF and heparin-binding EGF (HB-EGF) were significantly increased in sarcoma subjects (p = <0.0001 and 0.0002, respectively), whereas TGF-α showed no statistical difference. Urokinase plasminogen activator (uPA), primarily responsible for the cleavage of plasminogen to plasmin, was decreased in sarcoma subjects (p = 0.0008); this sharply contrasted with the increased levels of plasminogen activator inhibitor-1 (PAI-1) (p = <0.0001), a known antagonist of uPA (Fig. 1A). The proinflammatory cytokines IL-6 (p = 0.0467), IL-8 (p = <0.0001), and MIP-1β (also known as CCL4) (p = <0.0001) were elevated in our sarcoma subjects as compared with healthy controls. Additionally, the Th2-associated cytokine IL-4 was increased in sarcoma subjects relative to healthy controls (p = 0.0018). No differences were detected in levels of TNF-α, IL-18, or IL-12 (Fig. 1B). Thus, the presence of a localized sarcoma triggers a complex shift in the systemic host environment toward one that is favorable for continued tumor progression.

Obesity is associated with decreases in key Th2-related cytokines, but numerous protumorigenic proteins remain largely unaltered in the context of obesity

Because it is still not known to what extent obesity alters sarcoma progression or outcome, we investigated if increasing BMI had an impact on the systemic levels of tumorigenic and inflammatory markers in our sarcoma subject cohort. To accomplish this, we divided our cohort into three different groups based on BMI status following BMI classification as detailed by the World Health Organization's guidelines: normal weight was defined as having a BMI score ≤24.9 kg/m^2, overweight was defined as BMI score of 25–29.9 kg/m^2, and obese was defined as a BMI score ≥30 kg/m^2. As shown in Table II, we had an approximate 50–50 split of males and females of our sarcoma subjects in each BMI category (normal weight: 50% female, 50% male; overweight: 60% female, 40% male; and obese: 48% female, 52% male). Due to the complex subtyping of sarcoma, we again had differential representation of various subtypes within these groups. In our healthy controls, demographics were normal weight: 75% female, 25% male; overweight: 36% female, 64% male; and obese: 47% female, 53% male. The average age and SD of our sarcoma subjects was 50 ± 28, 60 ± 17, and 60 ± 18 y of age in normal weight, overweight, and obese subject groups, respectively. Similarly, the average age and SD of our healthy controls was 53 ± 10, 59 ± 13, and 51 ± 15 y of age in normal weight, overweight, and obese subject groups, respectively. When we examined angiogenic factors, we surprisingly found that there were no statistical differences in any of these analytes (VEGF-A/C/D, placental growth factor, angiopoietin-2) when stratified by BMI status in sarcoma subjects. This lack of alteration in the presence of obesity was mirrored throughout many of the additional analytes examined (endoglin, PAI-1, uPA, etc.) (Fig. 2A). Remarkably, we determined that only soluble CD40 ligand (sCD40L, also known as soluble CD154) [ANOVA, p = 0.0130; post hoc p = 0.0114 (normal to overweight), p = 0.0313 (normal to obese)], and EGF [ANOVA, p = 0.0115; post hoc p = 0.0096 (normal to overweight), p = 0.0440 (normal to obese)] were significantly altered in the presence of obesity; both analytes were significantly reduced in overweight and obese groups as compared with their normal-weight counterparts (Fig. 2A). Thus, in our sarcoma subject cohort, the systemic protumorigenic serum protein profile was relatively unchanged by increasing BMI. Interestingly, there were no detectable differences in any of

the aforementioned analytes tested in our healthy control group when stratified by BMI, suggesting that alterations in sCD40L and EGF occur only in the presence of combined overweight or obesity and sarcoma growth.

Inflammation is recognized as a hallmark of cancer (17), and it has notable roles in the pathogenesis of obesity. As such, we sought to examine the potential changes in proinflammatory serum cytokines and chemokines in our normal weight, overweight, and obese sarcoma subjects. We found that obesity resulted in significant decreases in IL-7, IL-8, and MCP-1/CCL2 (IL-7 ANOVA $p = 0.002$, IL-8 $p = 0.020$, CCL2 $p = 0.027$) (Fig. 3A). Surprisingly, we noticed that there were overall alterations in systemic Th2-related cytokines with obesity. There was an overall trending decrease of IL-4 in overweight and obese groups compared with the normal weight category when grouped as a whole ($p = 0.0690$), and there were also significant decreases in IL-13 in obese subjects compared with overweight subjects (Fig. 3A). However, we found that decreasing IL-4 levels correlated with increasing BMI ($p = 0.0169$) when examined by linear regression analyses, a trend also seen for IL-7 and IL-13 ($p = 0.0188$, 0.0422, respectively) (Fig. 3C). IL-5 also showed a similar profile, as there were significant decreases in serum IL-5 in the obese group compared with the normal weight group ($p = 0.0031$) (Supplemental Fig. 1A), with this decrease also correlating with increasing BMI (Supplemental Fig. 1B). Th2 cells can also produce IL-10, a known immunoregulatory cytokine; however, we found no change in IL-10 in either our sarcoma subjects or healthy controls in the presence of obesity (Fig. 3B, Supplemental Fig. 1A). This finding suggests that the presence of obesity negatively impacts some, but not all, Th2-related cytokines. The Th1-related cytokine, IFN-γ, was also significantly decreased in obese subjects compared with normal-weight subjects, and these decreasing levels correlated with increasing BMI (Supplemental Fig. 1A, 1B); IL-12p70, however, had only a trending decrease in obese subjects as compared with overweight subjects, and this change did not reach significance (Fig. 3A). Again, we saw no alterations of these cytokines (including Th2- and Th1-related cytokines) in our healthy controls (Fig. 3B). Interestingly, we did identify an increase inMIP-1β (CCL4) in healthy controls when stratified by BMI; the magnitude of this change is minor compared with the change in concentration of MIP-1β (CCL4) of sarcoma subjects as a whole versus healthy donors (34.5× higher in sarcoma subjects than healthy donors). These data support the idea that many of the cytokine and chemokine alterations we detected are found only when obesity is present as a comorbidity in the context of sarcoma growth.

Overall, our data reveal that although numerous serum proteins are altered in response to localized sarcomas, many of these responses are not significantly altered by comorbidity with obesity in treatment-naive subjects. One notable exception is the family of protumorigenic Th2-related cytokines, which appear to be reduced in obese sarcoma subjects relative to normal-weight sarcoma subjects. This finding may serve as a potential avenue for the development of immunotherapeutic interventions for sarcoma patients.

DISCUSSION

In this study we present a comprehensive profiling of systemic cytokine and chemokine and protumorigenic factors in treatment-naive sarcoma subjects. We identified 15 inflammatory

or protumorigenic proteins that were significantly altered in the serum of our sarcoma subjects as compared with healthy donors. The net result is a skewing of the systemic host environment toward one that is favorable for tumor progression. We also examined the impact of obesity (as defined by BMI) on the systemic cytokine, chemokine and protumorigenic protein profiles in our sarcoma subject cohort. Obesity is a major healthcare concern in the United States, and with rates of adult obesity predicted to exceed 40% by 2030 (6), the numbers of sarcoma patients with obesity at diagnosis will likely increase. The presence of obesity as a comorbidity may be particularly important in the context of immunotherapy for sarcoma, as evidence from multiple preclinical studies has shown that obesity can promote tumor-derived immune suppression and negatively impact immunotherapeutic efficacy (12–16). Our current study shows that six of the proteins examined were significantly decreased in sera from sarcoma subjects whose BMI was ≥30 kg/m^2 (obese), relative to sarcoma subjects with BMI ≤ 24.9 kg/m^2 (normal weight). This surprising result suggests that obesity in sarcoma patients may not present an additional barrier to immunotherapeutic efficacy. In particular, the decreased systemic Th2-cytokine responses (IL-4, IL-5, IL-13) seen with increasing BMI may actually be a positive factor in terms of immunotherapeutic outcomes, as Th2 responses are known to impair protective $CD8^+$-mediated tumor immunity (18, 19). Collectively, our results illustrate that although sarcoma growth stimulates a protumorigenic systemic host environment, this is not exacerbated by the presence of obesity.

Many immunotherapies currently on the market or in development seek to enhance $CD8^+$ T cell–mediated tumor eradication, and the $CD4^+$ T cell compartment plays critical roles in shaping the scope and quality of the $CD8^+$ response. We found multiple changes in cytokines associated with T cell–based immune responses in sarcoma subjects in the presence versus absence of obesity, particularly related to the hallmark Th2 cytokines IL-4, IL-5, and IL-13. Although associations between Th2 immunity and sarcoma progression or response to immunotherapy have not been thoroughly evaluated, at least one prior report indicated that a strong Th2 cytokine profile correlated with poor response to therapy and heightened recurrence in children with soft tissue sarcoma (20). In addition, Hosoyama et al. (21) found that IL-4 and IL-13 increased tumoral outgrowth of human and mouse rhabdomyosarcoma cell lines, and that administration of IL-4R–blocking Abs in a mouse model of alveolar rhabdomyosarcoma attenuated metastasis and increased overall survival. Interestingly, the investigators commented on the potential role of IL-4 and IL-13 secretion by $CD4^+$ Th2 cells to activate tumor-associated macrophages, which in turn induce metastasis through tumor-associated macrophage–mediated secretion of EGF (21). However, the authors did not directly test this possibility in their studies. We determined that both IL-4 and EGF were increased systemically in our sarcoma subject cohort relative to healthy controls. However, we found that as BMI increased, systemic concentrations of Th2 cytokines IL-4, IL-5, and IL-13 significantly decreased ($p = 0.011$, 0.017, 0.003, and 0.042, respectively), as did those of EGF ($p = 0.0440$) (Figs. 2, 3, and Supplemental Fig. 1). Although these relationships were statistically significant, the small r^2 values ($r^2 = 0.11$, 0.17, 0.08, respectively) indicate that only 8–17% of the variability in individual serum cytokine concentrations can be explained solely by increasing BMI. This suggests that other factors, such as sarcoma subtype or comorbidities like diabetes might also be influencing the

observed responses. Nevertheless, given the heterogeneity in our subject cohort as related to the sarcoma subtypes represented, these significant decreases in Th2-associated cytokine concentrations are striking. IL-10 is another immunomodulatory cytokine that can be secreted by Th2 cells. In contrast to IL-4, IL-5, and IL-13, the concentrations of IL-10 were not altered by being overweight or obese. This suggests that obesity selectively impacts the production of hallmark Th2 cytokines. One possible explanation is that cytokine secretion by IL-10–producing T regulatory cells in sarcoma subjects is less affected by obesity than is cytokine secretion by Th2 cells. This idea would require validation in a prospective study.

When we examined sarcoma subjects relative to healthy controls, we found numerous proteins systemically expressed at higher concentrations in the presence of sarcoma. Notably, we determined that the expression of MIP-1β, commonly known as CCL4, was 34.5× higher in sarcoma subjects than in healthy controls. Although CCL4 was unchanged by obesity status in sarcoma subjects, it did increase with obesity in healthy controls; however, these alterations were dwarfed by the amount of CCL4 present in sarcoma subjects. Currently little is known regarding the mechanisms by which CCL4 might influence sarcoma progression or regression. A study by Flores et al. (22) evaluated pediatric and adolescent subjects with osteosarcoma and found that high levels of CCL4 differentiated osteosarcoma subjects from controls, and that high circulating CCL4 significantly correlated with increased overall survival. Although our study was not centered on pediatric patients (the mean age of our sarcoma subjects was 58 y), it may be beneficial to determine the potential of CCL4 to act as a biomarker or therapeutic target in adults with sarcoma. This is particularly relevant given the substantial and clear divergence in serum CCL4 concentrations in sarcoma subjects versus healthy controls (with means of 974 pg/ml versus 28.2 pg/ml, respectively). Prior studies in other tumor models have described CCL4 expression as being either tumor promoting (23–25) or being host protective and associated with prolonged survival (26, 27). Therefore, an understanding of the exact role of CCL4 in adult sarcoma is needed before its use as a potential therapeutic target can explored.

Cancer biomarkers are consistently sought after for their value in predicting patient outcomes and in utilization for therapeutic intervention strategies, and our study reveals several sarcoma-specific candidates that should be considered for future evaluation. For example, we found that serum PAI-1 was increased in our sarcoma subject cohort (an average of 11× greater than controls) (Fig. 1). In contrast, uPA was decreased relative to healthy controls. Both uPA and PAI-1 have been explored previously in sarcoma subjects. PAI-1 expression was found to be higher in primary tumors, whereas uPA mRNA was shown to be higher in metastases. Associations between tumor volume and PAI-1 expression in osteosarcomas have been described, as have as correlations of uPA or PAI-1 with tumor invasion (28). uPA and PAI-1 have been examined for potential use as prognostic biomarkers of lymph node–negative breast cancer (29), suggesting that these proteins may also serve as useful and novel biomarkers in sarcoma patients. In addition, our analyses indicate that both EGF and HB-EGF are present systemically at higher concentrations in sarcoma subjects than healthy controls (Fig. 1). HB-EGF has been identified previously as a potential biomarker in certain subtypes of sarcoma (30), and high levels of HB-EGF transcripts have been correlated with decreased overall survival (31). EGF is expressed in soft-tissue sarcomas, and initial studies into EGFR blockade plus chemotherapy have shown decreased

tumor growth both in vitro and in a mouse model of fibrosarcoma (32), but EGFR levels may contribute to starvation- and chemotherapeutic-resistance in osteosarcoma (33). EGFR expression is highly variable in human sarcoma tumors and cell lines (32–34), indicating that therapeutic EGFR targeting may have to be determined on a case-by-case basis. Nevertheless, our results suggest that both EGF and PAI-1 should be investigated further as therapeutic targets in sarcoma subjects.

sCD40L has been shown to be substantially increased in osteosarcoma, chondrosarcoma, and Ewing sarcoma patients (35, 36). Our data agree with these findings, as we identified an increase in sCD40L in our sarcoma subject cohort. To the best of our knowledge, intervention strategies targeting sCD40L in human sarcoma have not been investigated. Recent investigations into high serum sCD40L in subjects with tumors have revealed that CD40-CD40L interactions may function as a double-edged sword in cancer immunity, as sCD40L was found to mediate immunosuppressive effects, including the expansion of regulatory T cells (37), whereas CD40-CD40L interactions facilitate the induction of protective $CD8^+$ T cell–based immune responses. Therefore, interventions targeted to this pathway should be approached with caution, as this therapeutic approach may hinder beneficial immune responses.

A prior report by Rutkowski et al. (38) showed that multiple cytokines (IL-1RA, sIL-2Rα, IL-6, IL-8, IL-10, TNF-α, TNFR1, TNFRII,M-CSF, bFGF, and VEGF) were elevated in pretreatment sarcoma subjects as compared with healthy donor controls (n = 156 patients, 50 controls), but this study did not examine potential effects of obesity on serum protein profiles. The Rutkowski study found that serum levels of IL-6 and IL-8 were correlated with tumor grade whereas serum levels of IL-6, sIL-2R, VEGF, M-CSF, and TNF-RI were correlated with tumor size. Interestingly, IL-1RA, IL-6, IL-8, IL-10, TNFRIII, and M-CSF were significantly decreased in subjects' serum postintervention (38). We also found systemic increases in serum IL-6 and IL-8 in our treatment-naive sarcoma subjects; however, we are unable to make any conclusions about the systemic levels of these cytokines post-therapeutic intervention. Furthermore, we are unable to make any conclusions about our serum cytokine concentrations in relation to tumor grade, as all of our subjects had high-grade tumors. Rutkowski et al. (38) also determined that higher serum levels of IL-6 were independently correlated with decreased disease-free survival postsurgery. In light of these data, further investigations regarding therapeutic targeting of these inflammatory IL signaling cascades may be warranted.

Despite the numerous novel findings we describe in this study, we recognize that there are limitations. For example, we used only BMI score as a measure of obesity status. There have been multiple recent reports that BMI may not be the most ideal measurement of body composition; hence, other obesity metrics, such as waist circumference or dual-energy x-ray absorptiometry scanning, may give a more in-depth understanding of obesity and its effects on tumor immunity in sarcoma patients (39–41). Furthermore, we had a limited number of samples (n = 50) and included multiple sarcoma subtypes in our cohort, likely increasing variability in our results. The fact that we identified multiple significantly up- or downregulated serum proteins in the presence or absence of sarcoma and obesity suggest that these changes are broadly applicable to many sarcoma subtypes. This conclusion should

be verified in larger cohort studies. Furthermore, this study was exclusively limited to serum factors and did not examine cellular factors such as infiltrating immune cells or expression of proteins commonly targeted by immunotherapeutic Abs (such as programmed cell death protein1, programmed death ligands 1 and 2 or CTLA-4 expression, among others). Future studies should include such metrics to comprehensively examine the broader impacts of being overweight or obese on immunotherapeutic outcomes in sarcoma subjects. Additionally, our biospecimens were all collected by the MAST registry at the University of Iowa. Thus, our results may reflect serum profiles only in the demographic population of that state, rather than addressing potential differences that may arise in a different geographical location with an entirely different demographic distribution.

To the best of our knowledge, we present in this study the first comprehensive systemic cytokine, chemokine, and protumorigenic factor profiling of treatment-naive human sarcoma patients as stratified by obesity status. This study has high clinical impact due to the progressive nature of the obesity epidemic, particularly as it relates to treatment of cancer patients with targeted biologics or immune-stimulatory therapies. We have identified increases in specific cytokines and chemokines, such as CCL4, that were not previously known to be systemically increased in adults with sarcoma. Additionally, we have provided a baseline profiling of protumorigenic, and cytokine and chemokine serum levels in the presence of an obesogenic environment. With further investigations, our findings may lead to new, more efficacious therapeutic interventions for sarcoma patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

BMI	body mass index
EGF	epidermal growth factor
HB-EGF	heparin-binding EGF
MAST	Melanoma and Sarcoma Tissue Bank
PAI-1	plasminogen activator inhibitor-1
sCD40L	soluble CD40 ligand
uPA	urokinase plasminogen activator

VEGF vascular endothelial growth factor.

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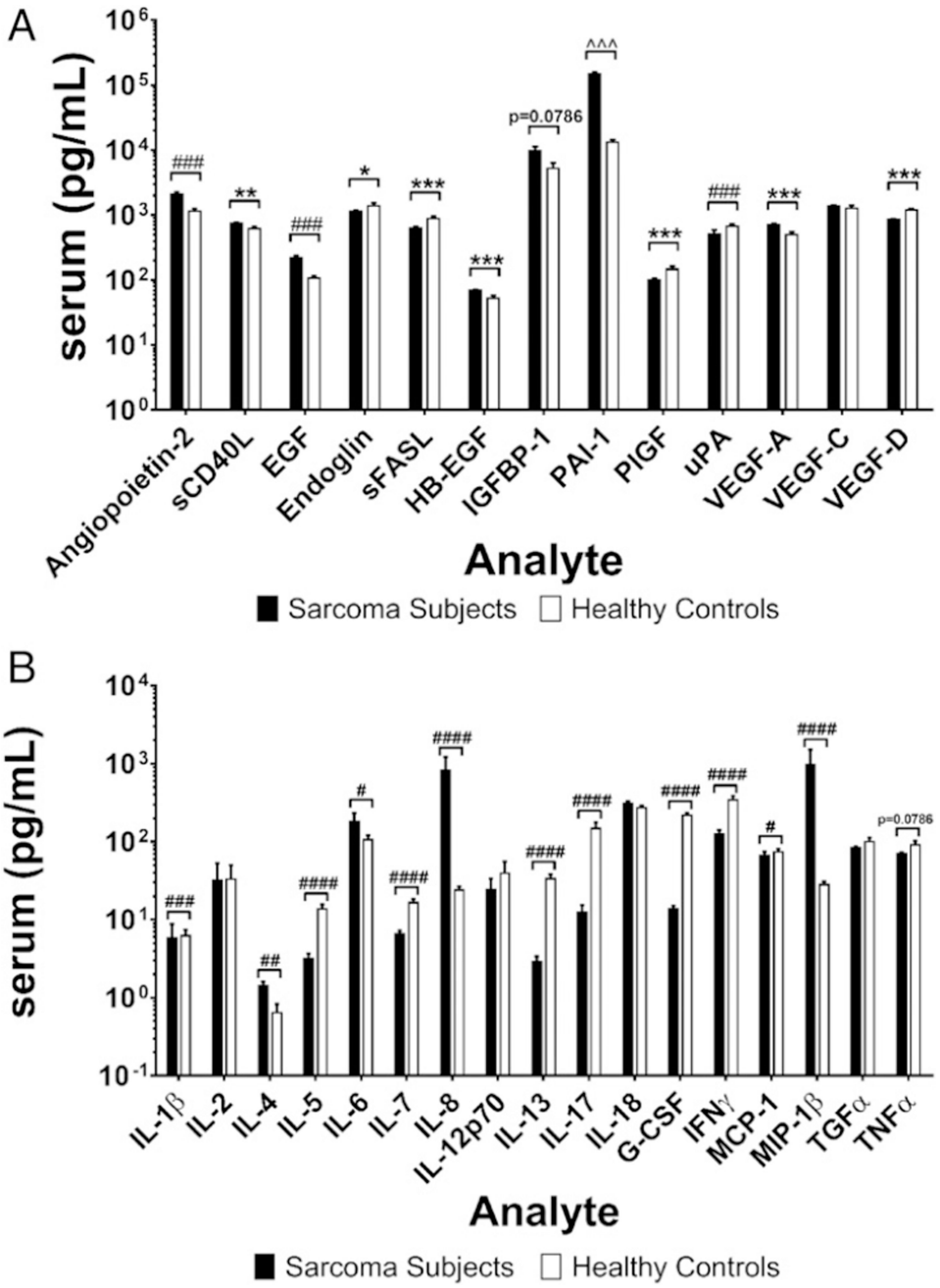

FIGURE 1. Systemic cytokine and chemokine profiling of sarcoma subject serum reveals numerous alterations in analyte expression as compared with healthy donor controls

Banked human serum was obtained from sarcoma subjects and healthy donors. Quantitative analyte expression of serum samples was analyzed by Luminex-based assays for (**A**) human cancer biomarkers or (**B**) cytokines and chemokines. Gaussian distribution was assessed using the D'Agostino–Pearson omnibus normality ($p > 0.05$ threshold). Statistical significance was determined by either unpaired *t* tests with (as denoted by carets; ^) or without Welch correction (as denoted by asterisks; *), or by unpaired Mann–Whitney *U* test (as denoted by number/pound signs; #). *,#$p < 0.05$, **,##$p < 0.01$, ***,^^^,###$p < 0.001$, ####$p < 0.0001$.

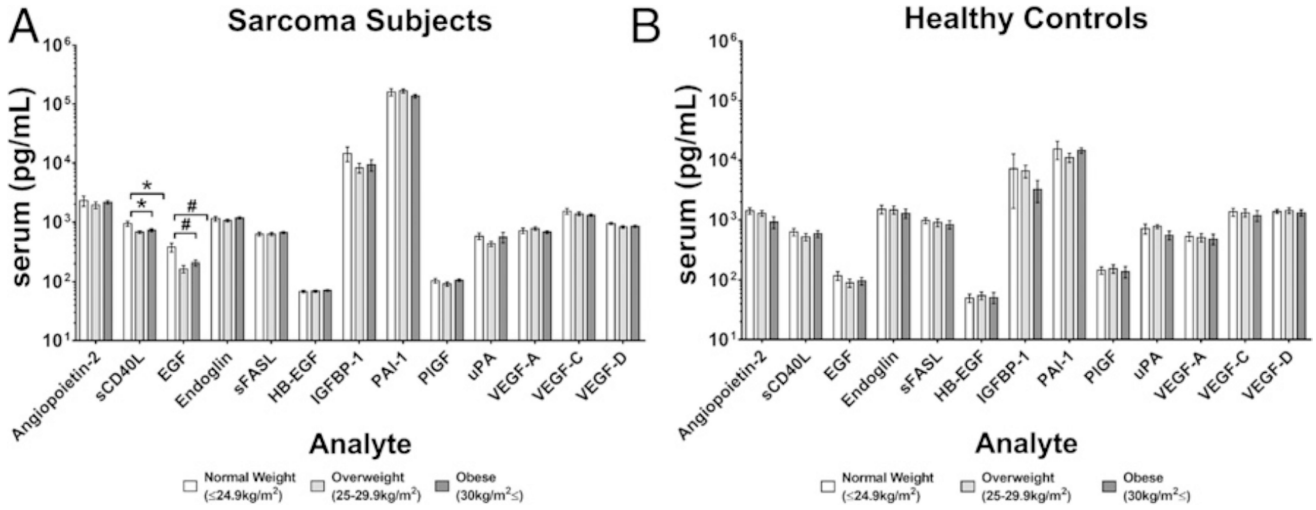

FIGURE 2. Numerous protumorigenic proteins are largely unaltered by obesity

Banked human serum was obtained from sarcoma subjects and healthy donors. Quantitative analyte expression of serum samples was analyzed by Luminex-based assays for human cancer biomarker, cytokines and chemokines. Analytes were then analyzed according to BMI grouping (normal weight ≤24.9 kg/m^2; overweight = 25–29.9 kg/m^2; obese ≥30 kg/m^2) for (**A**) sarcoma subjects alone or (**B**) healthy controls alone. Gaussian distribution was assessed using the D'Agostino–Pearson omnibus normality ($p > 0.05$ threshold). Statistical significance was determined by an ordinary one-way ANOVA with post hoc Tukey multiple comparisons test (as denoted by asterisks; *) or by Kruskal–Wallis test with post hoc Dunn multiple comparisons test (as denoted by number/pound signs; #). $^{*,\#}p < 0.05$.

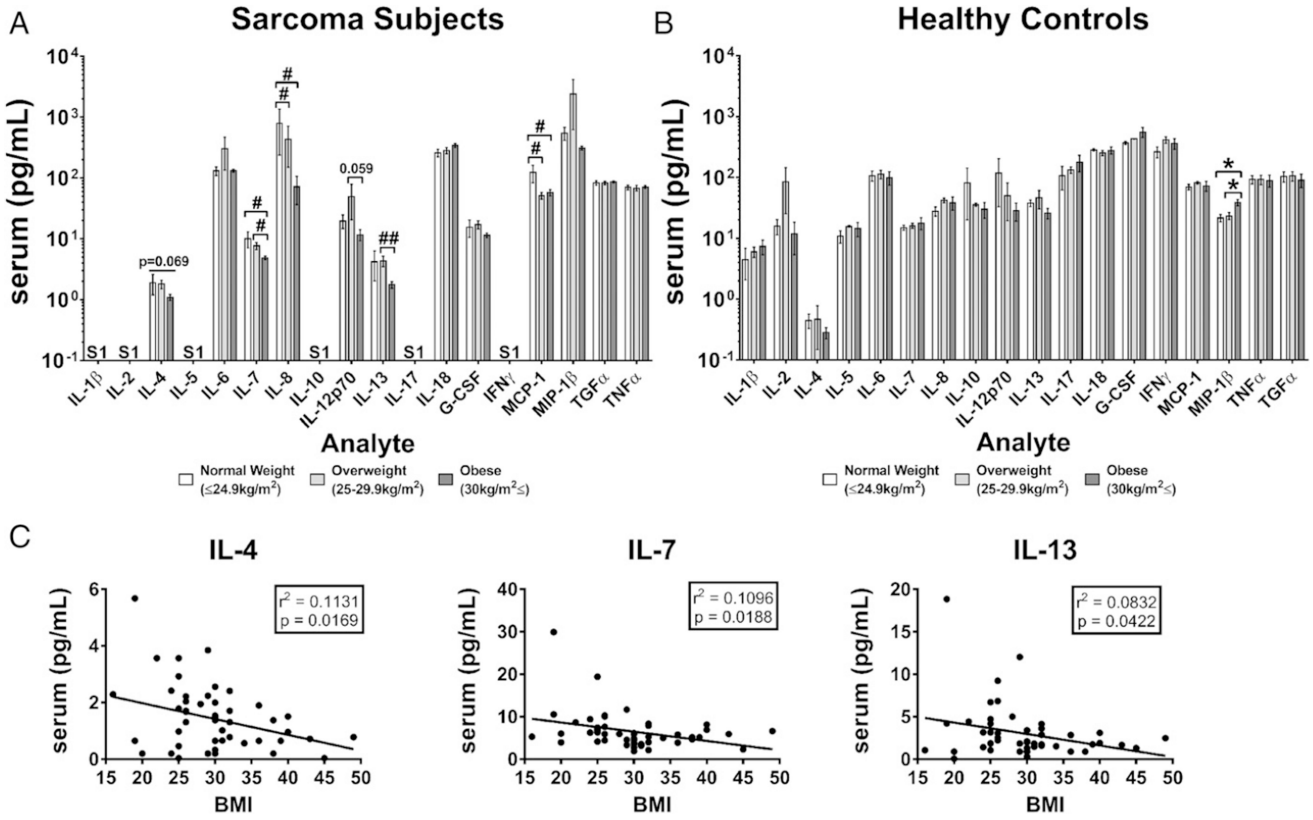

FIGURE 3. Obesity associates with decreases in serum Th2-related cytokines whereas numerous other cytokines and chemokines are largely unaltered in the presence of obesity

Banked human serum was obtained from sarcoma subjects and healthy donors. Quantitative analyte expression of serum samples was analyzed by Luminex-based assays for human cancer biomarker and cytokine/chemokines. Analytes were then analyzed according to BMI grouping (normal weight ≤24.9 kg/m^2; overweight = 25–29.9 kg/m^2; obese ≥30 kg/m^2) for (**A**) sarcoma subjects alone or (**B**) healthy controls alone. Those values that did not meet our analyte call threshold, as described in the *Materials and Methods* section, can be found in Supplemental Fig. 1, and are denoted by the S1 designation within the graphs in (A). Gaussian distribution was assessed using the D'Agostino–Pearson omnibus normality ($p > 0.05$ threshold). Statistical significance was determined by an ordinary one-way ANOVA with post hoc Tukey multiple comparisons test (as denoted by asterisks; *) or by Kruskal–Wallis test with post hoc Dunn multiple comparisons test (as denoted by number/pound signs; $^{\#}$). $^{*,\#}p < 0.05$, $^{\#\#}p < 0.01$. (**C**) Analyte concentrations were plotted out against corresponding BMI score and linear regression analyses were conducted. Statistical significance is indicated within the figure.

TABLE I

Patient demographics and tumor characteristics

Variable	Sarcoma Subjects	Healthy Donor Controls
Age (mean, y)	58.3	54.1
Sex		
Male	24 (48%)	17 (48.6%)
Female	26 (52%)	18 (51.4%)
Histological type		
Liposarcoma	2 (4%)	N/A
Leiomyosarcoma	10 (20%)	N/A
Pleomorphic sarcoma	7 (14%)	N/A
Myxofibrosarcoma	6 (12%)	N/A
Giant cell sarcoma	1 (2%)	N/A
Malignant fibrous histiocytoma	2 (4%)	N/A
Synovial sarcoma	2 (4%)	N/A
Rhabdomyosarcoma	1 (2%)	N/A
Sarcoma, NOS	3 (6%)	N/A
Peripheral nerve sheath tumor	3 (6%)	N/A
Osteosarcoma/chondrosarcoma	13 (26%)	N/A
Grade		
High grade	47 (94%)	N/A
Gr 2/3	3 (6%)	N/A

N/A, not applicable; NOS, not otherwise specified.

TABLE II

Subject characteristics by BMI category

Variable	Normal Weight (≤24.9 kg/m^2) *n* = 8	Overweight (25–29.9 kg/m^2) *n* = 15	Sarcoma Subjects: Obese (≥30 kg/m^2) *n* = 27	Sarcoma Subjects: Normal Weight (≤24.9 kg/m^2) *n* = 8	Sarcoma Subjects: Overweight (25–29.9 kg/m^2) *n* = 11	Healthy Controls: Obese (≥30 kg/m^2) *n* = 15
Gender						
Female	4 (50%)	9 (60%)	13 (48%)	6 (75%)	4 (36%)	7 (47%)
Male	4 (50%)	6 (40%)	14 (52%)	2 (25%)	7 (64%)	8 (53%)
Age (mean ± SD, y)	50 ± 28	60 ± 17	60 ± 18	53 ± 10	59 ± 13	51 ± 15
Histological type						
Liposarcoma	0 (0%)	0 (0%)	2 (7%)	N/A	N/A	N/A
Leiomyosarcoma	0 (0%)	4 (27%)	6 (22%)	N/A	N/A	N/A
Pleomorphic sarcoma	1 (13%)	2 (13%)	4 (15%)	N/A	N/A	N/A
Myxofibrosarcoma	0 (0%)	1 (7%)	5 (19%)	N/A	N/A	N/A
Giant cell sarcoma	0 (0%)	0 (0%)	1 (4%)	N/A	N/A	N/A
Malignant fibrous histiocytoma	0 (0%)	0 (0%)	2 (7%)	N/A	N/A	N/A
Synovial sarcoma	0 (0%)	1 (7%)	1 (4%)	N/A	N/A	N/A
Rhabdomyosarcoma	0 (0%)	1 (7%)	0 (0%)	N/A	N/A	N/A
Sarcoma, NOS	2 (25%)	1 (7%)	0 (0%)	N/A	N/A	N/A
Peripheral nerve sheath tumor	3 (38%)	0 (0%)	0 (0%)	N/A	N/A	N/A
Osteosarcoma/chondrosarcoma	2 (25%)	5 (33%)	6 (22%)	N/A	N/A	N/A
Grade						
High grade	8 (100%)	14 (93%)	25 (93%)	N/A	N/A	N/A
Grade 2/3	0 (0%)	1 (7%)	2 (7%)	N/A	N/A	N/A

N/A, not applicable; NOS, not otherwise specified.