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PRINCIPAL INVESTIGATOR: Jaime F. Modiano, VMD, PhD

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14. ABSTRACT Osteosarcoma is a cancer that primarily affects children, adolescents, and young adults. So far, treatment of this cancer with immunotherapy has been unrewarding. However, the mechanisms that mediate resistance to immunotherapy are poorly understood. Our goal is to determine whether osteosarcoma-derived exosomal microRNAs inactivate immune cells in or near the tumor environment. We have found that CD28 might be a target through which osteosarcomas restrain T cell activation, although different mechanisms might mediate this effect in tumors from humans and from dogs. Overall, the transcriptional programs in osteosarcoma, including those of immune cells in the microenvironment, are controlled by epigenetic mechanisms that include chromatin modification (methylation) and microRNA-dependent RNA degradation. A major innovation from our work is the development of a novel molecule that simultaneously inhibits myeloid and exhaustion immune checkpoints, and which may have the capacity to mitigate osteosarcoma-mediated immunosuppression. This approach could change the paradigm of how immunotherapy is used for this and other solid tumors.					
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1. INTRODUCTION:

Osteosarcoma is a cancer that primarily affects children and adolescents and young adults (AYAs). Despite intense, debilitating treatments, more than 50% of patients die within 10 years of diagnosis, making safe and effective treatments a critical unmet need for osteosarcoma. Osteosarcoma appears to be amenable to immunotherapy, but there are no methods to identify patients who might benefit from this approach because mechanisms of resistance to immunotherapy are incompletely understood. Our objective is to characterize mechanisms that mediate sensitivity and resistance of osteosarcoma to immune-based therapies. Specifically, we will examine the potential for osteosarcoma cells to package and secrete microRNAs (or other molecules) in exosomes which inactivate innate or induced host anti-tumor immune responses.

2. KEYWORDS:

Osteosarcoma, T-cells, CD28, immune checkpoints, exosomes, RNA, microRNA, next-generation sequencing, immunotherapy resistance, immune-related adverse events, animal models.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The project had *three specific aims*, each consisting of several tasks and subtasks. The aims and milestones are listed below. Tasks and subtasks with details regarding accomplishments are detailed in the next section, including modifications or deferral due to negative results (*i.e.*, failure to reject the null hypothesis).

Specific Aim 1: To determine the prevalence of CD28-targeting microRNAs in osteosarcoma and osteosarcoma-derived exosomes using a multi-species comparative approach.

Milestone: Protocol approvals by local IACUC/IRB and by ACURO/HRPO (Months 0-3). Done.

Milestone: Establish osteosarcoma molecular subclassification using evolutionarily conserved microRNA clusters. Done.

Milestone: Refine molecular subclassification of osteosarcoma based on correlated, evolutionarily conserved mRNA and microRNA clusters in cell lines, tumor tissues, and lymph nodes. Modified (lymph node sequencing was limited to assessment of clonality).

Milestone: Characterize immune infiltrates and their local and regional influence on disease progression based on conserved microRNA clusters subclassification of osteosarcoma based on correlated, evolutionarily conserved mRNA and microRNA clusters. Done.

Milestone: Identify systemic, evolutionarily conserved mRNA and microRNA clusters that can be used to assign prognosis in exosomes derived from paired cell lines, tumor tissues, and lymph nodes. Modified (lymph node sequencing was limited to assessment of clonality).

Milestone: Confirm immune infiltrates and their relation to disease progression based on identification of individual cell types using immunohistochemistry. Deferred due to COVID-19.

Milestone: Data analysis; organization and conclusions. Ongoing.

Milestone: Quality control and quality assurance for data – manuscript preparation and submission. Done and ongoing.

Specific Aim 2: To establish mechanisms of CD28 silencing by osteosarcoma-derived exosomes.

Milestone: Define effect of osteosarcoma-derived exosomes on expression of CD28, CD80, CD86 and other immune signaling complex markers. Done.

Milestone: Define effect of osteosarcoma-derived exosomes on T-cell activation. Done.

Milestone: Define effect of osteosarcoma-derived exosomes on T-cell migration in vitro. Deferred.

Milestone: Data analysis; organization and conclusions. Ongoing.

Milestone: Quality control and quality assurance for data – manuscript prep and submission. Done and ongoing.

Specific Aim 3: To define the relationship between CD28 silencing and resistance to immune checkpoint blockade in pre-clinical osteosarcoma mouse models.

Milestone: Defining a safe and effective dose for stabilized sPD-1 in mice and its relationship to CD28 expression. Modified (see progress report from Year-1).

Milestone: Define effect of tumor-derived exosomes on T cell activation, migration and anti-tumor response in mice receiving checkpoint blockade. Modified (see progress report from Year-1).

Milestone: Data analysis; organization and conclusions. Ongoing.

Milestone: Quality control and quality assurance for project data – manuscript prep and submission. Ongoing.

Milestone: Planning for pediatric clinical trial planning (translation to humans). Ongoing.

What was accomplished under these goals?

Special emphasis notes to the DOD program staff and reviewers:

Note 1: Please see details regarding the impact of the COVID-19 pandemic on section 5.

Note-2: Some of the experiments included in this report were completed prior to the funding period with financial support from other grants and agencies. They have been reported to the relevant funders. They are included here to provide context, because the analysis and integration of these data into the overall project was possible only through the support this project provided for personnel time and effort, as well as through the generation of new data for the project. Experiments that were completed with support from other sources or prior to the start date of this project are clearly identified as such in this report.

Specific Aim 1: To determine the prevalence of CD28-targeting microRNAs in osteosarcoma and osteosarcoma-derived exosomes using a multi-species comparative approach.

For this aim, we confirmed and extended previous data showing that a limited number of transcriptional programs is able to reduce the complexity of osteosarcoma into defined groups with prognostic significance. While we were able to establish the presence of CD28-targeting microRNAs in the osteosarcoma microenvironment, we concluded that, unlike what has been observed in other tumors such as colorectal cancer, these microRNAs and their potential suppression of CD28 are not likely to be the principal mechanism of immunosuppression in osteosarcoma. Rather, the immunosuppressive barrier in osteosarcoma is most likely functioning by excluding immune cells from the tumor environment, although the immune response to tumors might be regulated by epigenetic methylation of DNA which in turn controls the establishment of key transcriptional programs in each of the tumor constituents.

An important aspect of our work was the demonstration that the etiology of canine and human osteosarcoma is distinct, so there are limitations in using dogs as a model to understand *causation* of this disease. However, the eventual organization of the tumors into “osteosarcoma tissues” in both species relies on building convergent structures with highly conserved cellular and spatial features and comparable transcriptional programs. This provides a foundation for the canine disease to serve as a robust model of tumor progression, and potentially to develop novel, safe and effective therapeutic interventions.

The transcriptional programs include co-regulated clusters of microRNAs and co-regulated clusters of genes, with various levels of interdependency. However, microRNAs do not appear to be the principal mechanisms through which the immune response to osteosarcoma is regulated. Nevertheless, our data suggest that exosome cargo can be delivered to heterologous (non-tumor) cells locally and at distant sites, and it can alter the behavior of stromal cells in the environment.

Specific Aim 2: To establish mechanisms of CD28 silencing by osteosarcoma-derived exosomes.

For this aim, we showed that exposure to osteosarcoma-derived exosomes *in vitro* can alter the function of stromal cells that are found in the tumor environment (fibroblasts and endothelial cells), and even impair certain aspects of T cell activation. But generally, the effect on T cells was modest, with no noticeable effects on CD28 expression or T-cell viability. Our data suggest that the ability to extrapolate data regarding exosome effects *in vitro* to the *in vivo* conditions is limited, as the contents of exosomes from cultured cells (at least for mRNAs) are non-overlapping with the contents of exosomes from cells growing in the orthotopic site *in vivo*.

Specific Aim 3: To define the relationship between CD28 silencing and resistance to immune checkpoint blockade in pre-clinical osteosarcoma mouse models.

For this aim, we leveraged an opportunity to combine the design of a stabilized soluble PD-1 (sPD-1) molecule that could be used to block the interaction between surface PD-1 and its ligands, PD-L1 and PD-L2, with a peptide that would also block the interaction between CD47 (the prototypical receptor that provides a “do not eat me” signal) and its SIRPα ligand in

phagocytes. We manufactured the dual function blockade peptide in an *E. coli* inclusion body system and documented its capability to compete with antibodies that bind PD-L1 and CD47 in tumor cells from multiple species. We also showed that CD47 blockade and PD-1 blockade have therapeutic activity in mouse models, alone and in combination with other immune therapies. The peptide is actively undergoing pre-clinical development in mouse osteosarcoma models in combination with an oncolytic virus. These experiments are preamble to a preclinical study to examine the safety and efficacy of this combination therapy in dogs with metastatic osteosarcoma (funded through a DOD IMPACT grant).

Specific Aim 1: To determine the prevalence of CD28-targeting microRNAs in osteosarcoma and osteosarcoma-derived exosomes using a multi-species comparative approach.

Specific Aim 1, Task 1: Approval from HRPO and from ACURO

- University of Minnesota IACUC protocol 1803-35710A, was approved on April 12, 2018.
- ACURO approval for Proposal Number CA170218, Award Number W81XWH-18-1-0068 was obtained in September 18, 2018.
- On March 29, 2018, the University of Minnesota IRB determined that the proposed activity is not research involving human subjects as defined by DHHS and FDA regulations.
- On August 18, 2018, an HRPO Concurrence Memorandum (Proposal Number CA170218) was received, indicating the proposed activities did not constitute research involving human subjects.

Specific Aim 1, Task 2: Define osteosarcoma subtypes by microRNA clusters.

For this task, we obtained next-generation sequencing data (mRNA and small RNA) from an independent cohort of dogs with spontaneous appendicular osteosarcoma and used our datasets to define the existence of a limited number of interdependent transcriptional programs that can be used to define prognostic groups for osteosarcoma.

Specific Aim 1, Task 2, Subtask 1: Identify 50 non-duplicative, well annotated canine osteosarcoma samples (complete history, standard of care treatment, known disease-free interval and survival time) from our archive for next generation sequencing of small RNAs.

We obtained samples from 107 individual cases banked by the Canine Comparative Oncology and Genomics Consortium (CCOGC, Inc.). We triaged samples to include only those for which there was >50 mg of tissue. We further restricted the eligibility for the experiment to samples from appendicular sites, to reduce the heterogeneity of the set. Sixty-two (62) samples were selected for the sequencing project (**Table 1**). The population demographics for these samples (breed, age, gender) were consistent with expectations. We isolated total RNA from each of the 62 samples with the objective to prepare independent sequencing libraries for mRNA and for small RNA sequencing, allowing us to determine replication of our previous data in an independent set.

Table 1. Demographic Data and Metrics for RNA Extraction from Canine Osteosarcoma Samples

Sample ID	Breed	Site	Age (yrs.)	Sex	Nucleic Acid (ng/μl)	A260	A280	A260/280	A260/230
CCB010057	Labrador Retriever	Tibia	9	Female	160.7	4.018	1.94	2.07	1.29
CCB010058	Labrador Retriever	Tibia	10	Male	388.2	9.705	4.878	1.99	1.63
CCB010059	Golden Retriever	Tibia	12	Male	184.3	4.608	2.221	2.07	1.79
CCB010089	Rottweiler	Femur	8	Male	151.9	3.798	1.871	2.03	1.49
CCB010092	Staffordshire Bull Terrier	Femur	7	Female	1091.1	27.277	13.04	2.09	2.07
CCB020039	Great Dane	Radius	5	Male	338.6	8.466	4.038	2.1	2.06
CCB020056	Greyhound	Femur	8	Male	914.9	22.874	11.033	2.07	2.05
CCB020063	Greyhound	Humerus	8	Male	985.5	24.637	11.692	2.11	2.13
CCB020064	Greyhound	Humerus	6	Female	136.8	3.421	1.666	2.05	1.96
CCB020071	Golden Retriever	Radius	7	Male	85	2.125	1.055	2.01	2.1
CCB020078	Mixed Breed	Radius	8	Female	408.1	10.201	4.998	2.04	2
CCB020081	Golden Retriever	Humerus	8	Female	442.6	11.064	5.309	2.08	2.05
CCB020082	Labrador Retriever	Humerus	9	Female	111.2	2.781	1.363	2.04	1.81
CCB020083	Greyhound	Humerus	11	Male	367.3	9.183	4.378	2.1	1.77
CCB020085	Greyhound	Humerus	6	Female	215.2	5.38	2.593	2.08	1.84
CCB020094	Greyhound	Femur	11	Male	179	4.474	2.144	2.09	1.73
CCB020096	Mixed Breed	Femur	7	Female	733.4	18.335	8.949	2.05	1.81
CCB020108	Greyhound	Femur	6	Female	827.2	20.679	9.749	2.12	2.11
CCB020110	Mixed Breed	Ulna	10	Male	723.3	18.082	8.648	2.09	1.66
CCB020116	Mixed Breed	Tibia	5	Female	503.7	12.593	6.093	2.07	2.2
CCB020120	Greyhound	Femur	5	Male	447.8	11.194	5.341	2.1	2.13
CCB020143	Greyhound	Tibia	8	Male	412.3	10.308	4.916	2.1	1.92
CCB020156	Golden Retriever	Radius	9	Male	473.2	11.829	5.669	2.09	1.7
CCB020239	Old English Sheepdog	Radius	8	Male	318.8	7.97	3.795	2.1	1.95
CCB020248	Greyhound	Humerus	10	Male	2381.7	59.542	28.319	2.1	2.16
CCB020265	Old English Sheepdog	Tibia	8	Male	84.2	2.104	1.035	2.03	1.57
CCB030080	Mixed Breed	Ulna	2	Female	1563	39.075	18.458	2.12	2.18
CCB030081	Great Pyrenees	Radius	8	Female	480.5	12.013	5.889	2.04	2.09
CCB030096	Labrador Retriever	Radius	7	Female	663.5	16.587	7.923	2.09	2.17
CCB030104	Irish Wolfhound	Radius	6	Male	585.7	14.642	6.985	2.1	2.19
CCB030111	Labrador Retriever	Tibia	11	Female	98.1	2.453	1.215	2.02	1.37
CCB030116	Irish Wolfhound	Radius	5	Male	90.8	2.27	1.142	1.99	3.17
CCB030122	Weimaraner	Femur	10	Male	522.4	13.061	6.412	2.04	2.51
CCB030129	Mixed Breed	Humerus	11	Male	1225.9	30.646	14.5	2.11	2.14
CCB030143	Labrador Retriever	Radius	4	Male	102.8	2.57	1.317	1.95	1.89
CCB030153	Labrador Retriever	Humerus	12	Male	299	7.474	3.561	2.1	1.37
CCB030159	Boxer	Radius	10	Male	198.5	4.962	2.397	2.07	1.73
CCB030175	Great Dane	Radius	5	Male	471.4	11.786	5.593	2.11	2.15

Sample ID	Breed	Site	Age (yrs.)	Sex	Nucleic Acid (ng/μl)	A260	A280	A260/280	A260/230
CCB030198	Australian Cattle Dog	Femur	11	Female	191.4	4.786	2.315	2.07	2.09
CCB030221	Labrador Retriever	Radius	12	Male	355.1	8.878	4.262	2.08	2.12
CCB030306	Mixed Breed	Ulna	12	Male	570.3	14.258	6.693	2.13	2.15
CCB030328	Greyhound	Femur	8	Male	729.3	18.231	8.63	2.11	2.03
CCB030334	Labrador Retriever	Femur	11	Female	385.8	9.645	4.588	2.1	2.11
CCB040155	Mixed Breed	Ulna	12	Female	346.8	8.671	4.124	2.1	2.11
CCB050001	Mixed Breed	Femur	7	Female	272.7	6.817	3.238	2.11	2.11
CCB050015	Golden Retriever	Humerus	6	Male	1592.6	39.814	18.916	2.1	2.15
CCB050020	Doberman Pinscher	Humerus	11	Female	750.6	18.764	8.786	2.14	2.12
CCB060038	Rhodesian Ridgeback	Other Site	11	Male	494.6	12.366	6.046	2.05	2.21
CCB060058	Australian Shepherd	Femur	10	Female	116.1	2.904	1.408	2.06	1.91
CCB060083	Mixed Breed	Femur	10	Female	475	11.875	5.67	2.09	1.66
CCB070015	Belgian Sheepdog	Radius	6	Male	143.9	3.598	1.776	2.03	1.73
CCB070022	Irish Wolfhound	Radius	6	Female	1339.2	33.48	16.183	2.07	2.05
CCB070023	Rottweiler	Humerus	11	Female	440.9	11.022	5.407	2.04	1.91
CCB070068	Rottweiler	Femur	4	Female	498.5	12.463	6.272	1.99	2
CCB070157	Labrador Retriever	Radius	6	Male	229.4	5.735	2.745	2.09	1.99
CCB070212	Golden Retriever	Humerus	8	Male	180.8	4.521	2.181	2.07	1.16
CCB080001	Great Pyrenees	Tibia	10	Female	1006.3	25.157	11.848	2.12	2.14
CCB080003	Labrador Retriever	Femur	13	Male	294.9	7.372	4.064	1.81	1.03
CCB080010	Mixed Breed	Humerus	10	Male	158.6	3.966	1.91	2.08	1.99
CCB080013	Mixed Breed	Tibia	6	Female	864	21.6	10.598	2.04	1.74
CCB080031	Great Dane	Femur	6	Female	353.1	8.827	4.22	2.09	1.33
CCB080035	Labrador Retriever	Humerus	12	Female	418.9	10.472	4.998	2.1	2.15

Specific Aim 1, Task 2, Subtask 2: RNA isolation from tumor samples.

All of the initial RNA extractions passed quality control (QC) in our laboratory based on absorbance measures at ultraviolet wavelengths of 260 nm (A260), 280 nm (A280), and 230 nm (A230) (**Table 1**). These samples were submitted to the University of Minnesota Genomics Center (UMGC) in July of 2019. We expected sequencing to be completed by late September of 2019. However, we encountered obstacles with RNA integrity, which were compounded by the COVID-19 pandemic, which delayed completion of the sequencing experiments. Regarding lymph node samples, they were collected as part of a separate project supported through independent funding (from the State of Minnesota and Vyriad, Inc.). The main objective for the lymph node samples was to examine lymphocyte clonality in dogs after treatment with an oncolytic virus. Given budget constraints and the challenges of fitting work in during the COVID-19 pandemic, RNA sequencing of lymph node samples was not done.

Specific Aim 1, Task 2, Subtask 3: Nanoscale library preparation and next generation sequencing.

The RNA integrity numbers (RIN) obtained by staff at the UMGC using a Bioanalyzer TapeStation showed that, despite our solid QC numbers using absorbance, the RNA from the samples was of poor quality. We repeated RNA extractions on multiple samples until, by January of 2020, 43 of the 62 samples passed QC at the UMGC for mRNA sequencing based on bioanalyzer data and Ribogreen quantification, representing approx. 70% of the samples submitted (**Table 2**). We decided to move forward with these samples, as it was highly unlikely that the remaining samples would yield RNA of sufficient quality for sequencing. We should note, however, that this performance from primary bone samples was predictable, as extraction of intact, high quality RNA from primary bone or bone cancer samples is notoriously difficult. In the end, even though some of the 43 samples did not achieve the recommended RIN of 8.0, we sequenced them because in our experience, their RINs were still likely to yield good data. A total of 43 unique dual-indexed (UDI) TruSeq Stranded mRNA libraries were created. The libraries were pooled and sequenced on a NovaSeq S1 2x50-bp run. The sequencing run generated ≥ 750 million pass filter reads for the pool (approx. 17.5M reads per sample). All of the expected barcodes were detected and well represented. The mean quality scores for all libraries were $\geq Q30$. The libraries were gel size-selected to have inserts of approx. 200bp.

Table 2. Samples Sequenced for mRNA

Sample Name	Sample Concentration (ng/ul)	Total Mass (ng)	RIN
CCB010057	151.56	5288	6.2
CCB010092	255.97	5375	7.5
CCB020056	274.48	5764	8.3
CCB020063	148.29	3114	9.7
CCB020078	381.31	8008	6.1
CCB020081	230.33	4837	6.3
CCB020082	106.87	5579	7.0
CCB020083	281.71	5916	7.6
CCB020085	226.56	5674	8.2
CCB020094	182.95	5656	7.3
CCB020096	198.56	4170	8.3
CCB020108	378.72	7953	8.9
CCB020110	273.52	5744	8.1
CCB020116	542.67	11396	6.4
CCB020143	269.13	5652	6.9
CCB020156	295.41	6204	8.2
CCB020239	277.62	5830	7.6
CCB020248	188.01	3948	9.5
CCB030080	226.02	4746	8.5
CCB030081	188.03	3949	7.3
CCB030096	235.65	4949	7.8
CCB030104	233.15	4896	8.3

Sample Name	Sample Concentration (ng/ul)	Total Mass (ng)	RIN
CCB030122	331.51	6962	9.2
CCB030129	382.21	8026	9.5
CCB030143	222.85	12657	8.6
CCB030153	370.17	7774	6.6
CCB030175	272.08	5714	8.2
CCB030198	217.38	6229	6.7
CCB030221	235.37	4943	7.0
CCB030306	235.51	4946	8.5
CCB030328	217.92	4576	6.6
CCB030334	274.31	5761	8.7
CCB040155	279.31	5865	6.8
CCB050001	258.76	5434	7.2
CCB050015	423.11	8885	7.0
CCB050020	183.06	3844	6.0
CCB060038	340.10	7142	8.7
CCB060083	257.72	5412	8.7
CCB070015	141.89	5595	8.2
CCB070022	258.00	5418	8.5
CCB080001	333.91	7012	8.1
CCB080013	286.40	6014	9.4
CCB080031	224.07	4705	6.1

We also faced obstacles achieving the RNA quality needed for generation of small RNA libraries. In the end, after repeated attempts at extraction, only 28 of our samples passed QC at the UMGC because the input concentration needed for small RNA library preparation is higher than that needed for mRNA library preparation (**Table 3**). The samples also required further clean-up to eliminate genomic DNA carried over from the original RNA purification steps to ensure equal representation of the samples in the pooling step of the sequencing protocol, since small RNA library preparations do not include the polyA selection step that allows mRNA library preparation even with some degree of contaminating gDNA. Twenty-eight single indexed TruSeq smRNA libraries were created. The libraries were pooled and sequenced on a NextSeq Mid-output 2x75-bp run. The sequencing run generated approx. 120 million pass filter reads for the lane (approx. 4.5M reads per sample). All of the expected barcodes were present, but the balance was variable with high %CV. This is fairly typical of smRNA library pools as there are variable amounts and concentrations of smRNA at the target size going into size selection. The mean quality scores were $\geq Q30$ for all libraries.

Table 3. Samples Sequenced for Small RNA

Sample Name	Sample Concentration (ng/ul)	Total Mass (ng)	RIN
CCB010092	255.97	5375	7.5
CCB020056	274.48	5764	8.3
CCB020083	281.71	5916	7.6
CCB020085	226.56	5674	8.2
CCB020094	182.95	5656	7.3
CCB020096	198.56	4170	8.3
CCB020108	378.72	7953	8.9
CCB020110	273.52	5744	8.1
CCB020156	295.41	6204	8.2
CCB020239	277.62	5830	7.6
CCB030080	226.02	4746	8.5
CCB030081	188.03	3949	7.3
CCB030096	235.65	4949	7.8
CCB030104	233.15	4896	8.3
CCB030122	331.51	6962	9.2
CCB030129	382.21	8026	9.5
CCB030143	222.85	12657	8.6
CCB030175	272.08	5714	8.2
CCB030221	235.37	4943	7.0
CCB030306	235.51	4946	8.5
CCB030334	274.31	5761	8.7
CCB050001	258.76	5434	7.2
CCB050015	423.11	8885	7.0
CCB060038	340.10	7142	8.7
CCB060083	257.72	5412	8.7
CCB070022	258.00	5418	8.5
CCB080001	333.91	7012	8.1
CCB080013	286.40	6014	9.4

Specific Aim 1, Task 2, Subtask 4: Next generation RNA sequencing datasets of 44 human and 89 canine osteosarcoma-small RNAs will be mapped to the corresponding (human or canine) genome.

For this task, we used data from our previously sequenced canine and human osteosarcoma sample sets (performed with support from the Zach Sobiech Fund of the Children’s Cancer Research Fund and previously reported¹) and from the new canine osteosarcoma sample set described above. Sequence reads were mapped to the latest build of the canine and human genomes. Data from the more recently sequenced cohort of canine samples supported by this project and described in the subtasks above was mapped to CanFam3.1.

Specific Aim 1, Task 2, Subtask 5: Differentially expressed microRNAs will be identified based on variance and expression levels.

The work completed under this task was supported through the effort of key personnel in the project using sequence data that were available prior to the start of the project. Differentially expressed microRNAs clusters were identified for humans, including data on specific microRNAs that validated previous results from our laboratories and others²⁻⁵. The original canine dataset described above, which we call “DOS” was of low and high variable quality based on mapping percentages. However, the new sample set, which we call “CCB,” had higher percentages of reads mapped to microRNA sequences, indicating that it had higher quality of sequence. Analyses to compare the presence of conserved microRNA clusters in human and canine osteosarcoma are still ongoing, as the improved sequence from the new dataset might enable identification of sample clustering according to expression of distinct microRNA species. Please see the narrative at the end of Task 2, subtask 7 for a detailed description of the microRNA data in context with mRNA data, as well as the narrative for Task 4.

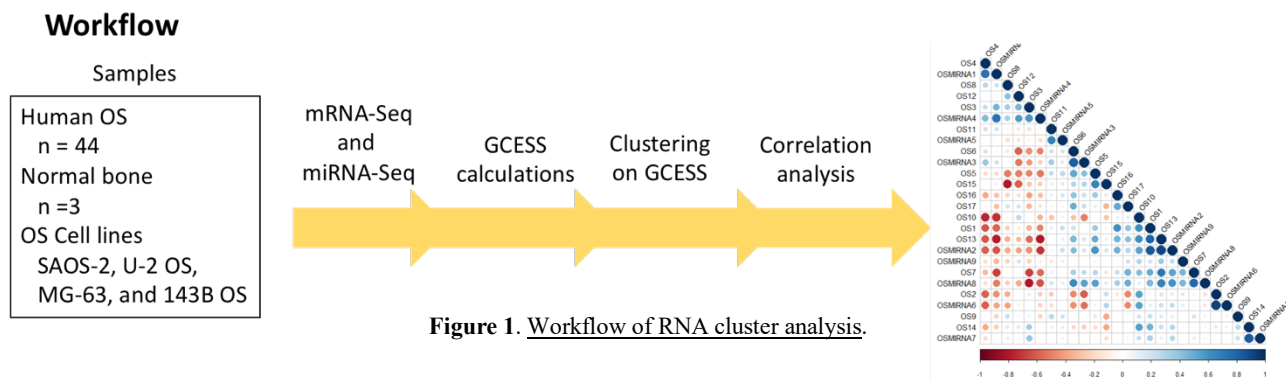
Specific Aim 1, Task 2, Subtask 6: Unsupervised hierarchical clustering will be used to define sample groups and microRNA clusters using our previously defined gene cluster expression summary scores (GCESS) methods.

Co-regulated microRNAs that could be defined into clusters using the GCESS method were identified. Please see the narrative at the end of subtask 7 for a detailed description of the microRNA data in context with mRNA data.

Specific Aim 1, Task 2, Subtask 7: Conserved clusters will be identified based on their location in syntenic blocks and sequence homology.

This summary details the identification of microRNAs clusters using the GCESS method and their relationship to mRNA clusters. These data are the mainstay of a manuscript that is in preparation for submission.

Workflow for data analysis (Specific Aim 1, Task 2, Subtasks 5-7): Next generation RNA sequencing data from 44 human osteosarcoma samples, three (3) samples of normal human bone, and four (4) human osteosarcoma cell lines are included in this analysis. Sample processing and analyses, including derivation of gene clusters and gene cluster expression summary scores (GCESS) were done as described^{1,6}. The workflow for correlation analyses is illustrated in **Figure 1**.



Correlation Analysis of RNA Clusters (Specific Aim 1, Task 2, Subtasks 5-7): Seventeen (17) independent mRNA clusters were identified in the human osteosarcoma dataset. The smallest cluster included 102 gene members and the largest cluster included 1,346 members. Nine (9) independent microRNA clusters were identified in the human osteosarcoma dataset. Pairwise correlation analyses were performed for all the clusters in tissue samples and cell lines together and separately (**Figure 2**). A functional annotation of each gene (mRNA) cluster and each microRNA family is shown in in **Table 4**.

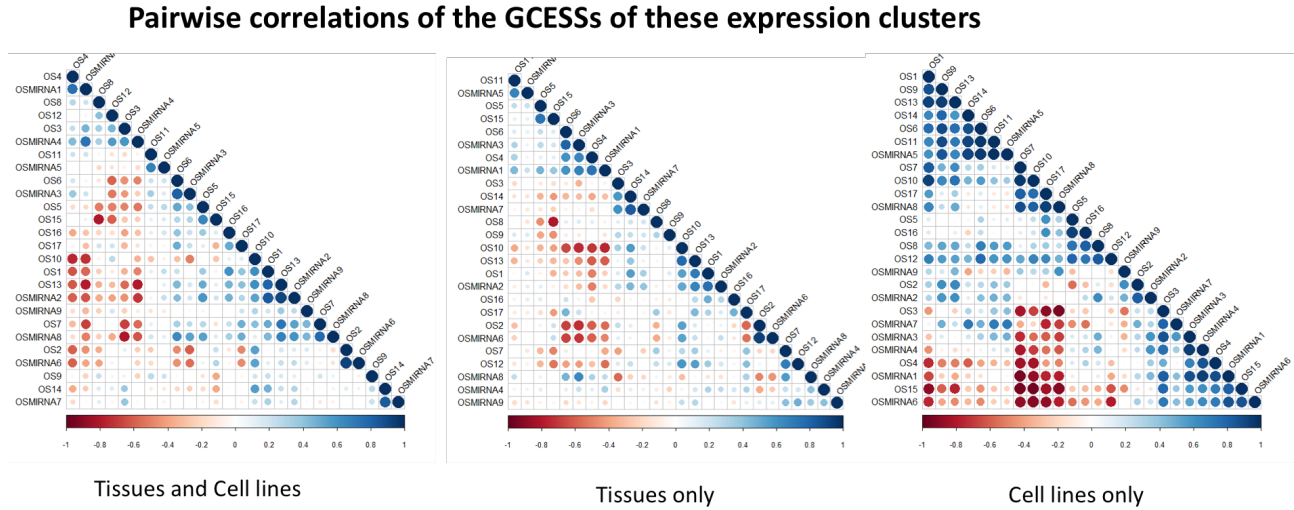


Figure 2. Pairwise correlation of GCESS for 17 mRNA clusters and nine (9) microRNA clusters.

In this analysis, blue color represents direct correlation ($r^2 > 0$), and red color represents inverse (or anti-) correlation ($r^2 < 0$). The size of the filled circles is proportional to the strength of direct or inverse correlation. As a control, the diagonal in each pairwise correlation analysis (same sample) is always equal to 1.0. It is worth noting that stronger correlations identified when the cell lines were analyzed independently were predictable, as these data represent RNAs and microRNAs from pure populations, all grown under the same laboratory conditions, thus harboring substantially less heterogeneity within and among samples.

We analyzed the correlation between individual mRNA clusters in osteosarcoma tissues and normal bone (excluding the cell lines). A matrix of all the correlations is shown in **Figure 3**. Significant correlations are illustrated in **Figure 4**. Consistent with predictions, the expression patterns of cluster OS1, which includes CD28 and CD86, and cluster 13, which includes CD80, were directly correlated ($r^2 = 0.70$, $p < 0.0001$). Ongoing analyses are validating that homologous relationships are conserved in osteosarcomas from dogs.

Next, we analyzed the correlation between individual microRNA clusters in osteosarcoma tissues and normal bone (excluding the cell lines). A matrix of all the correlations is shown in **Figure 5**. Significant correlations are illustrated in **Figure 6**. Analyses are ongoing to establish if homologous relationships are conserved in osteosarcomas from dogs.

Table 4. Annotation of gene (mRNA) and microRNA clusters.

A. mRNA Clusters	Functional Annotation	B. microRNA Clusters	microRNA Family
OS1	Immune cell signature	OSMIRNA1	17~92 cluster
OS2	Muscle signature	OSMIRNA2	
OS3	Neuronal	OSMIRNA3	mir-181 / miR-140 / mir-455
OS4	Cell cycle (MYC)	OSMIRNA4	
OS5	Blood cells (hemoglobin)	OSMIRNA5	mir-519 cluster
OS6	Bone development	OSMIRNA6	miRNA-1
OS7	Epithelial	OSMIRNA7	
OS8	Chemokine/hormone signaling	OSMIRNA8	
OS9	Tumorigenesis	OSMIRNA9	14q32 cluster
OS10	Tumorigenesis		
OS11	MAGEA / GABRA		
OS12			
OS13	Blood vessels		
OS14	Epithelial		
OS15	Cell death ... of osteoblasts		
OS16	Bone		
OS17	Connective tissue / movement		

Color coded clusters represent those that have been previously defined.

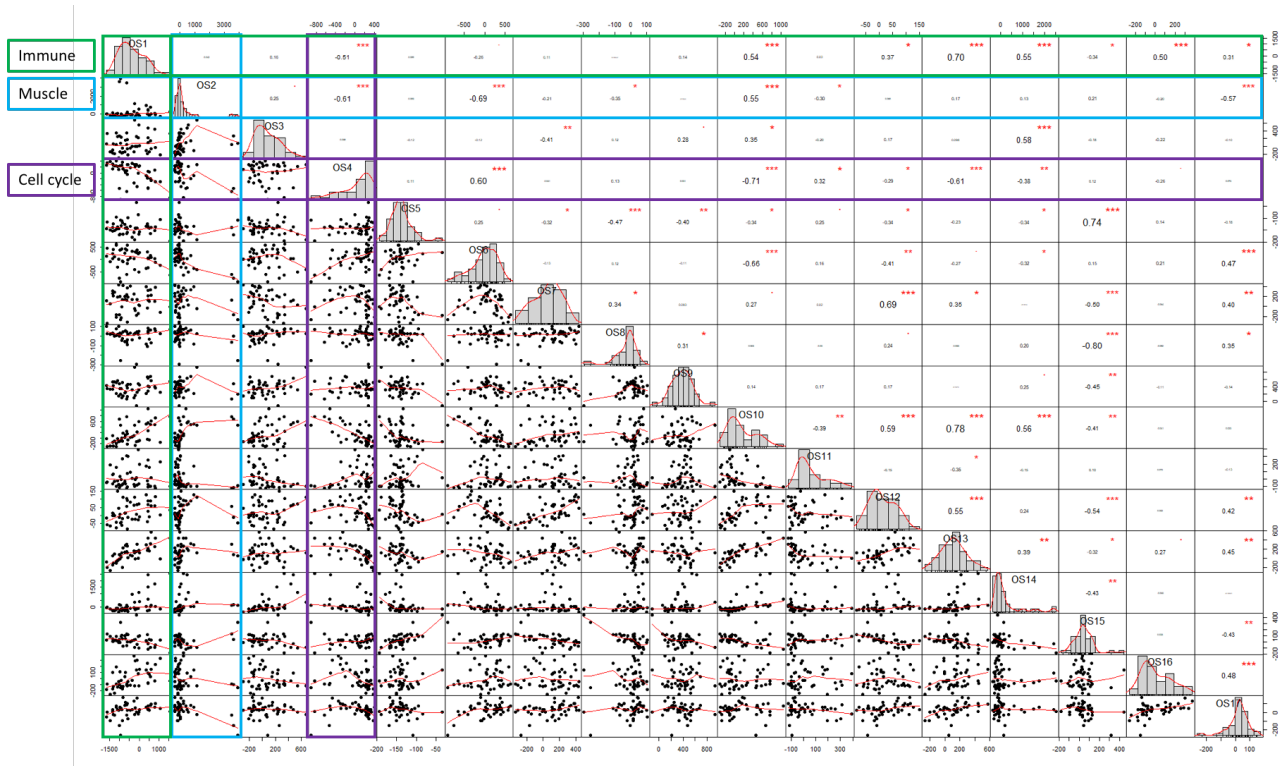


Figure 3. Matrix of all correlations for mRNA clusters in osteosarcoma tissues and normal bone samples.

Correlation of mRNA clusters in osteosarcoma tissues and normal bone samples

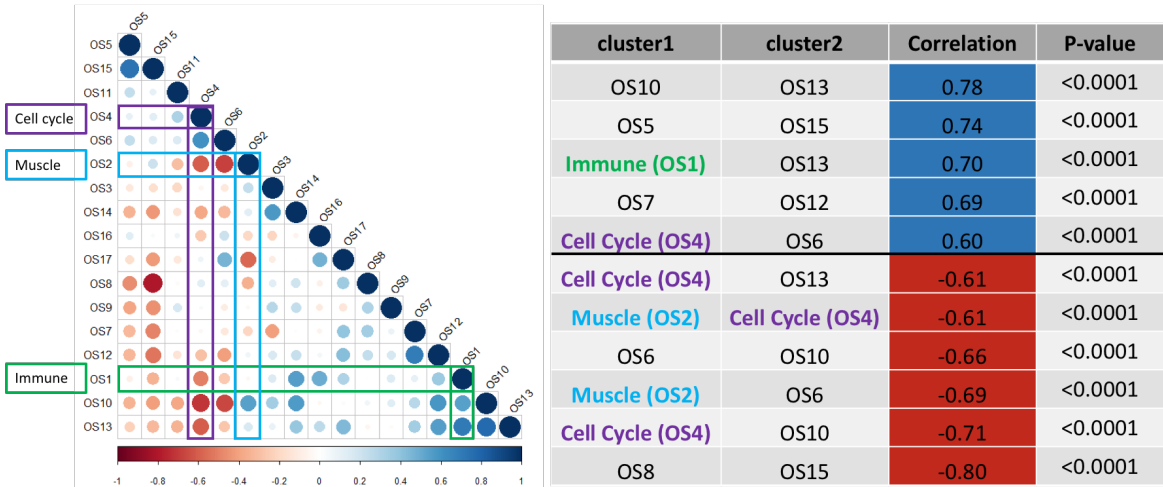


Figure 4. Correlation analysis for mRNA clusters in osteosarcoma tissues and normal bone samples. Significant positive and negative correlations are tabulated in the right panel of the figure, indicated by blue and red shading, respectively. The functional annotation of the Immune (OS1), Muscle (OS2) and Cell Cycle (OS4) clusters is indicated by name and color coding.

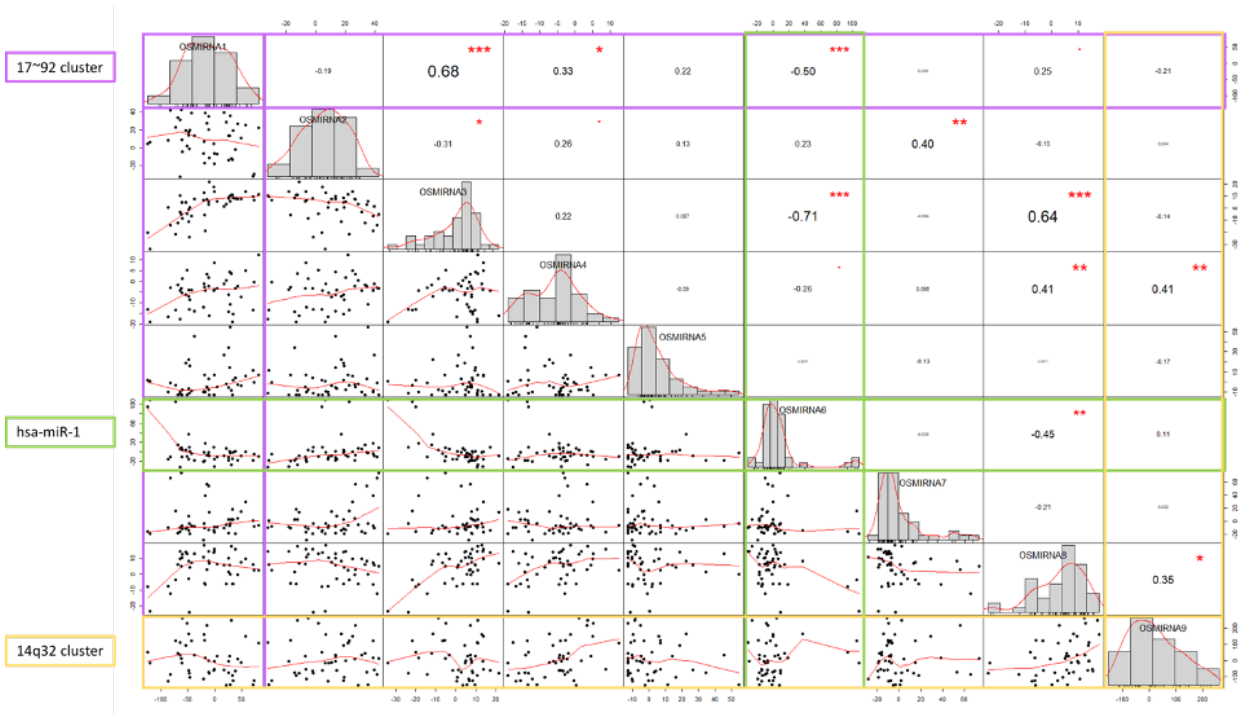


Figure 5. Matrix of all correlations for microRNA clusters in osteosarcoma tissues and normal bone samples.

Finally, for this analysis, we examined the correlation between individual mRNA clusters and microRNA clusters in osteosarcoma tissues and normal bone (excluding the cell lines). Significant correlations are illustrated in **Figure 7**. Consistent with our predictions, we identified significant, direct correlations between the hsa-miR-1 cluster and the muscle-associated mRNA signature ($r^2 = 0.94$), and between the miR-17~92 cluster and the cell cycle-associated mRNA

signature ($r^2 = 0.74$). To our surprise, and somewhat inconsistent with our previous data analyzing microRNA expression using conventional methods (not NGS)⁵, the miR-14q32 cluster was not correlated with the miR-17~92 cluster, or with the cell cycle-associated mRNA signature.

Correlation of miRNA clusters in OS tissues and normal bone samples

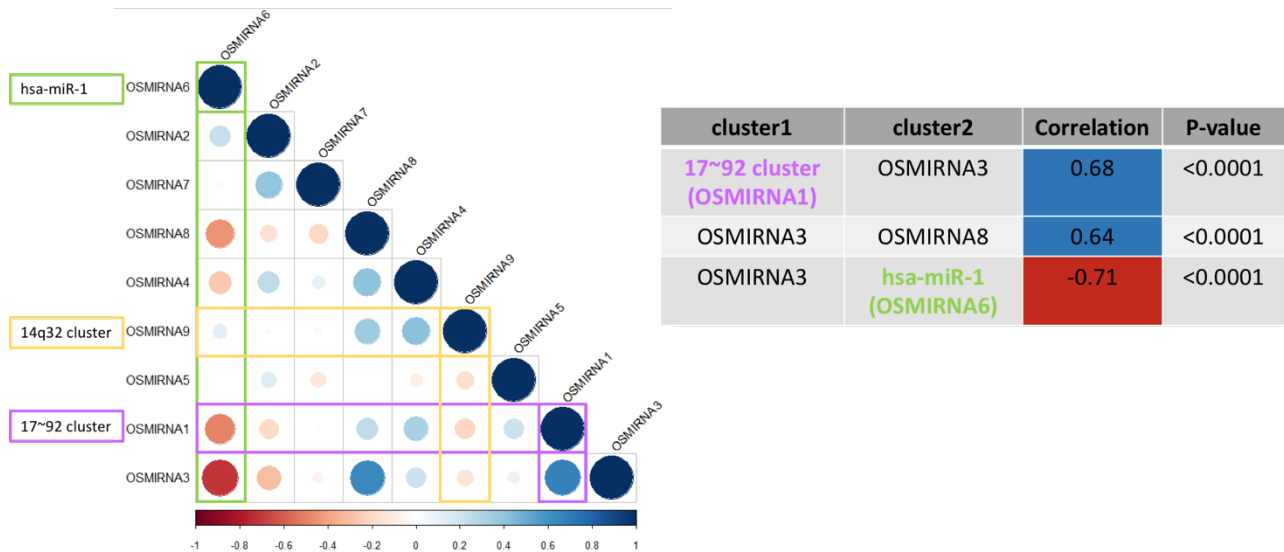


Figure 6. Correlation analysis for microRNA clusters in osteosarcoma tissues and normal bone samples. Significant positive and negative correlations are tabulated in the right panel of the figure, indicated by blue and red shading, respectively. MicroRNA families that include hsa-miR-1 cluster, the miR-14q32 cluster, and the 1miR-7~92 cluster are indicated by name and color coding.

Correlation of mRNA to miRNA clusters in OS tissues and normal bone samples

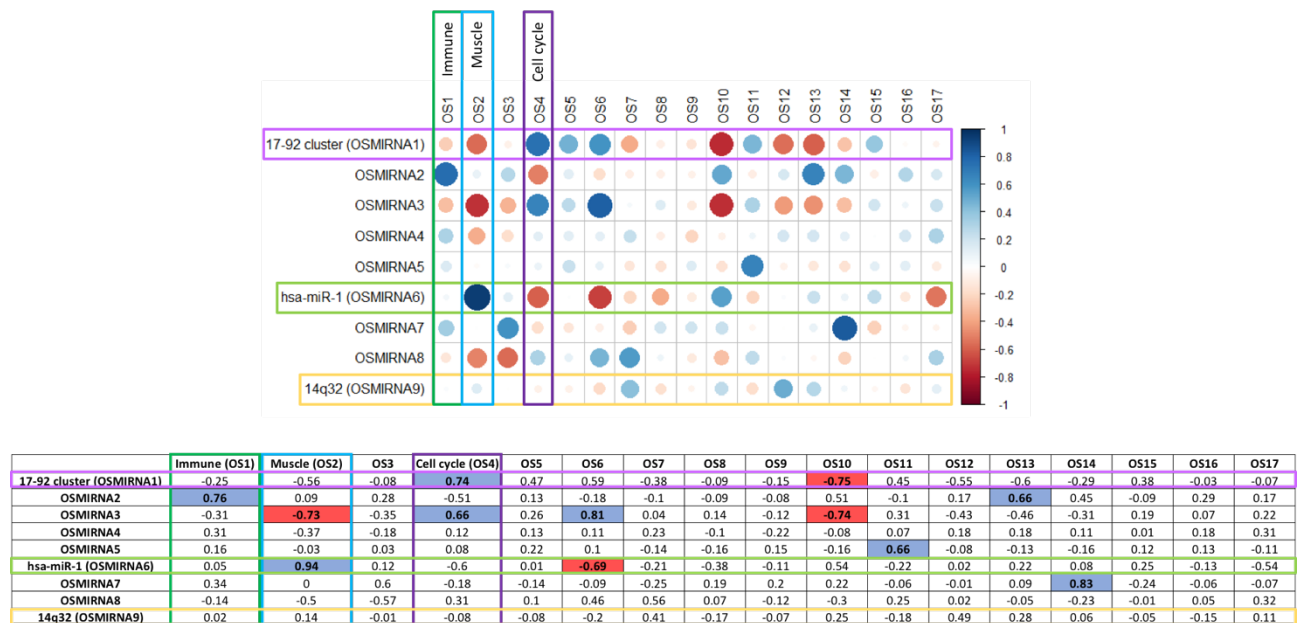


Figure 7. Correlation analysis for microRNA clusters in osteosarcoma tissues and normal bone samples. Significant positive and negative correlations are tabulated in the bottom panel of the figure, indicated by blue and red shading, respectively.

Specific Aim 1, Task 3: Establish if the molecular subclassification of osteosarcoma by microRNA clusters has prognostic value.

For this task, we showed that there was a correlation between the 19~92 microRNA cluster (OSMIRNA1) and the cell cycle transcriptional cluster, indicating that expression of 17~92 microRNAs is likely to be prognostically significant in humans and in dogs. Additional work to understand the relationships between microRNA clusters and mRNA clusters in dogs is still ongoing.

Specific Aim 1, Task 3, Subtask 1: Disease free interval and overall survival times will be evaluated statistically for sample groups defined by microRNA clusters as described above. Please see the narrative at the end of Task 3, subtask 2 below for details.

Specific Aim 1, Task 3, Subtask 2: Statistical analyses will use the Kaplan-Meier probability analysis and log-rank test.

The original DOS canine data set we sequenced included outcome information for approximately 20 dogs that had received standard of care therapy (amputation and adjuvant chemotherapy)¹. In that dataset, we observed that the cell cycle mRNA GCESS was inversely associated with survival in dogs and in humans with osteosarcoma (greater GCESS = shorter survival time), and that the two immune clusters were correlated and directly associated with survival in humans, although not in dogs¹. Several possible explanations could account for the difference between dogs and humans in this regard. The first possible explanation is that the relationship between immune GCESS and outcome in humans was spurious. This is unlikely, as we have established a similar relationship between immune GCESS and many other cancers in the TCGA¹. A second possible explanation is that the compressed progression of disease in dogs does not provide sufficient time for their immune system to mount an effective response. We also believe this is unlikely, given our current understanding of cancer and the adaptations of each species to an evolutionarily-determined aging program^{7,8}. A third possible explanation is that inbreeding in dogs has weakened their immune system and rendered it ineffective to fight tumors. This is not consistent with the natural history of disease as observed in dogs. In immunocompromised animals, tumors tend to occur earlier in life; unlike what is seen in dogs, where tumors, including osteosarcoma, tend to occur later in life. Moreover, unlike immunocompromised lab animals where immunotherapy (without immune cell transfer) relies exclusively on the activity of the innate immune system, dogs with osteosarcoma are capable of responding to therapies that activate the adaptive immune system. This observation that immunotherapies can be remarkably effective in companion dogs with osteosarcoma (regardless of breed background) strongly supports the conclusion that dogs have functional and competent immune systems⁹. A fourth, and perhaps most likely possibility, is that the heterogeneity of treatments received by the dogs, the reluctance of owners to continue treatment after metastasis is detected, and the small sample size, did not provide the power to detect a statistical correlation between the presence of immune cells (as measured by the quantitative immune GCESS) and outcome.

Each of the 43 dogs in the new CCB dataset that was sequenced for this project included treatment and outcome data. Yet, there were still significant differences in the standard of care used and in the owners' willingness to continue treatment of dogs after they relapsed. Seven dogs were treated by amputation alone. Five dogs received non-standard chemotherapy protocols (six

or more drugs or gemcitabine as the only chemotherapy drug) or investigational drugs (mTOR inhibitors or unnamed compounds under clinical investigation). The remaining 31 dogs were treated with surgery and adjuvant chemotherapy, but the chemotherapy protocols included doxorubicin alone (+/- suramin), carboplatin alone, combination doxorubicin and carboplatin, or combination carboplatin and gemcitabine. Some of these dogs received additional therapy after relapse (most often toceranib and/or pamidronate) and some received supplemental artemisin. Despite these differences, the disease-free interval and overall survival time (**Figures 8A and 8B, respectively**) for dogs treated with amputation (approximately 120 and 150 days, respectively), and with standard of care (approximately 180 and 240 days, respectively) were comparable to those that have been reported elsewhere¹⁰⁻¹⁶. Dogs in the experimental group might have exceeded the expected survival compared to those receiving standard of care, but the sample size was too small and diverse to reach a definitive conclusion. Ongoing analyses are evaluating correlations between GCESS (see below) and survival in this newly sequenced cohort of dogs with osteosarcoma.

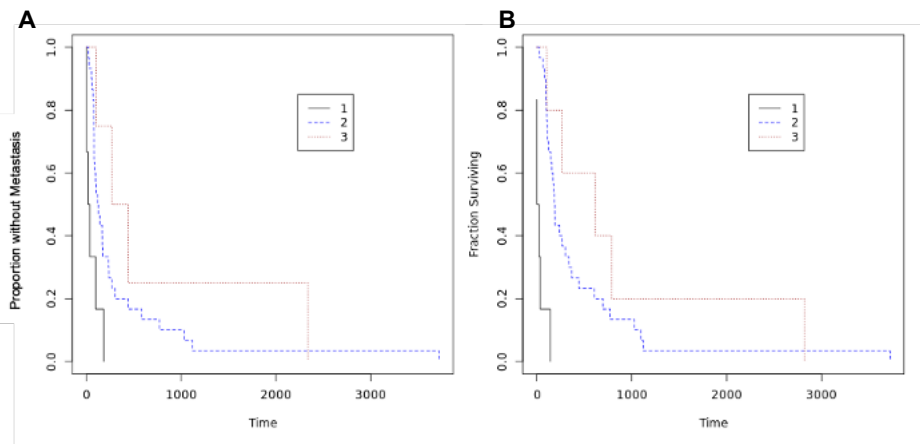


Figure 8. Kaplan Meier survival curves for new dataset of dogs with osteosarcoma. (A) Disease-free interval and (B) Overall survival time for dogs treated with surgery alone (group 1, solid grey line), with surgery plus adjuvant chemotherapy (group 2, dashed blue line), or with experimental therapies (group 3, red dotted line) as described in the text.

In previous work, we had shown that the expression of microRNAs from the 14q32 cluster (OSMIRNA1) was inversely correlated with the presence of metastasis at diagnosis (greater expression = increased probability of metastasis) and with time to metastasis in humans, as well as with overall survival time of humans and dogs⁴. Regrettably, the human osteosarcoma samples for which we completed next-generation microRNAs sequencing were not annotated for survival and there are no publicly available data to explore this correlation. However, based on the previously characterized relationships between the mRNA and microRNA clusters, and our published analysis of the relationship between cell cycle and immune clusters and outcome¹, we expect a prognostic relationship should be apparent. Such a correlation was not seen in the original DOS canine osteosarcoma dataset, but this could have been due to the quality of sequencing. Given the improved quality metrics achieved in the newly sequenced CCB cohort, we are continuing to evaluate potential relationships between microRNA clusters, mRNA clusters, and survival of dogs with osteosarcoma.

Specific Aim 1, Task 4: Define statistical correlations of microRNAs with immune transcript abundance.

For this task, we documented that a single, microRNA cluster was positively correlated with the immune gene clusters. As described in greater detail below, this suggests that transcriptional

regulation of immune-related genes is probably not mediated by microRNAs in osteosarcoma tissues. To explore the regulation of immune gene transcription further, we examined DNA methylation in subsets of our human and canine osteosarcoma samples (as part of a collaboration with Roche/Nimblegen) and showed that methylation modules were strongly correlated with the presence of immune transcripts. This suggests that transcriptional control (chromatin accessibility), rather than post-transcriptional regulation through microRNAs, might be a more relevant mechanism of immune gene regulation in osteosarcoma tissues. It is worth noting, however, that our data also suggest exclusion of immune cells from the osteosarcoma environment as a likely mechanism contributing to the osteosarcoma immunosuppressive barrier.

Specific Aim 1, Task 4, Subtask 1: Correlation analyses will be done between microRNA clusters defined in Task 2 and previously reported immune transcript clusters. Analysis will use one-to-one as well as one-to-many comparisons, since multiple microRNA families or microRNAs within a cluster might coordinately regulate stability of immune transcripts, and immune transcripts in each of the two clusters might be regulated by microRNAs in a single cluster.

Please see also related data under Specific Aim 2.

Our correlation analyses showed that the OSMIRNA2 was the only microRNA cluster that showed a significant association with the mRNA clusters derived from infiltrating (or resident) immune and inflammatory cells in osteosarcoma (**Figure 7**). These two clusters showed a positive (direct) correlation, indicating that microRNAs in the OSMIRNA2 cluster were unlikely to directly target immune transcripts for degradation. Work evaluating possible relationships between microRNA clusters and immune transcript abundance in the canine datasets is ongoing. However, given the lack of obvious correlations suggesting microRNAs as a major mechanism controlling the immune programs in osteosarcoma, we evaluated the possibility that these programs were regulated transcriptionally by alterations in chromatin accessibility (*i.e.*, DNA methylation).

Epigenetic regulation of the immune response to cancer can occur post-transcriptionally, for example, by destabilization of mRNAs that are essential to initiate or maintain anti-tumor immunity, or by alterations in the DNA and/or histone methylation landscape of immune cells within the tumor. We did not identify microRNA clusters that were significantly anti-correlated with immune response genes. Therefore, we examined osteosarcoma-associated methylomes in humans and dogs to understand whether the immune response might be regulated through methylation. We used the Epi-Giant kit to obtain high resolution, genome-wide methylation data for human samples, and we worked with Roche/Nimblegen to develop a new kit with capture baits to assess canine methylation at an average resolution of approximately 5 Mb. Methylation regions were clustered and compared against gene expression profiles. We also developed cell-type specific methylation profiles to quantify the abundance of cell types based on methylation patterns. The results from this work are published and available in the public domain^{17,18}. The manuscript published in the journal *Bone* is included as an appendix to this report.

Key data from this study that are most pertinent to this task show that there is a correlation between the immune transcriptional programs (as defined by immune GCESS) and methylation modules in both humans and dogs (**Figure 9**).

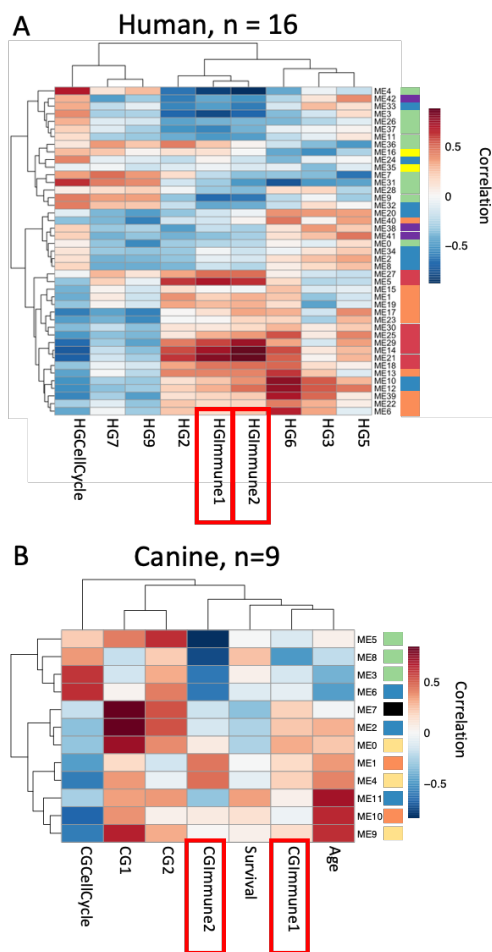


Figure 9. Correlation between methylation clusters and transcriptional programs in human and canine osteosarcoma. Correlations between methylation summary values for each DNA methylation cluster and previously described expression programs were calculated for human (A, n=16) (and canine B, n=9) samples where methylation and expression data were available. Correlations were also calculated for additional phenotypic data for canine samples. Note the green methylation modules are positively correlated with the cell cycle GCESS in both humans and dogs, while the orange methylation modules are negatively correlated in both. Conversely, the green methylation modules are negatively correlated with the immune GCESS (highlighted by red boxes) in both and the orange and red methylation are positively correlated with the immune GCESS. Heatmaps indicate Pearson correlation values between each DNA methylation cluster and phenotype where red is strong positive correlation and blue is strong negative correlation. Color bars next to module names indicate pathway analysis results and are the same as in the previous figure. HG = human GCESS, CG = canine GCESS(from ref. ⁵).

To summarize, our findings show that there are conserved modules of methylation in human and canine osteosarcomas. These methylation modules create patterns that are linked to key transcriptional programs in the tumors. In particular, the observed correlation between methylation and immune transcript abundance suggests that chromatin accessibility, may be the principal regulator of these transcriptional programs in osteosarcoma. This is consistent with the regulatory biology of immune cells and in turn, it suggests that the immunosuppressive barrier in osteosarcoma is created through other mechanisms, such as impeding entry of immune and inflammatory cells into the tumor environment, as shown clearly by the immunohistochemistry images in Scott et al¹ (**Figure 10**). Even though immune transcripts are readily detectable, this detection is probably due to the amplification of RNA signals (many transcripts in one cell).

In spite of apparent species differences, such conserved processes of gene regulation must reflect pathogenetically significant events that contribute to risk, progression, and therapeutic failure, and understanding their mechanisms will aid the development of better methods to identify risk and prognosticate progression, and ultimately more effective strategies for treatment, control, and eventually prevention.

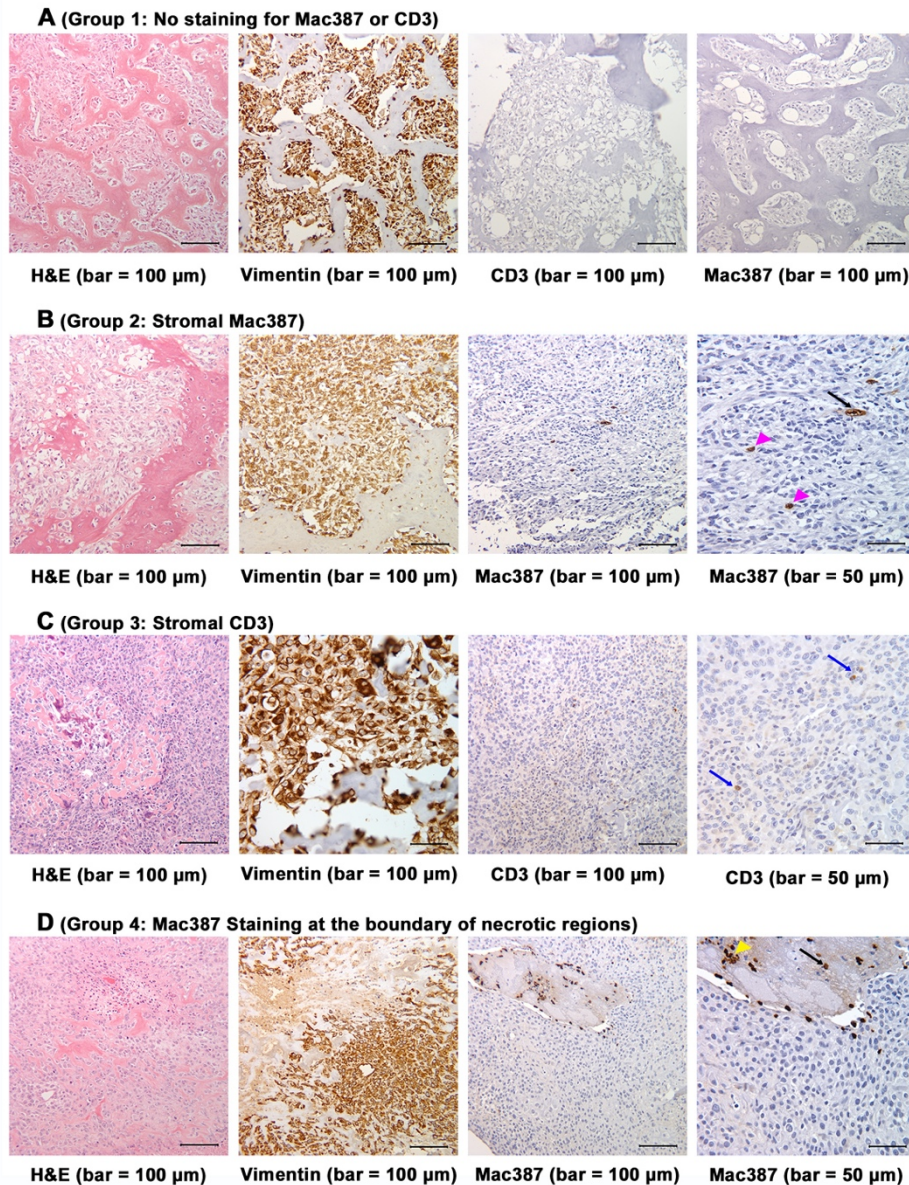


Figure 10. Examples of four groups of immune infiltrating cells in osteosarcoma. Representative images from canine osteosarcoma samples stained with hematoxylin and eosin (H&E), and with antibodies against vimentin, CD3, and Mac387 (S100A8/A9). (A) Samples from DOS-75, where tumor cells express vimentin uniformly, but there are no detectable T cells (no staining for CD3) or macrophages or granulocytes (no staining for Mac387). (B) Samples from DOS-115, where tumor cells express vimentin uniformly, and there are detectable myeloid cells within vessels (black arrow) and macrophages in the tumor stroma (red arrowheads). (C) Samples from DOS-101, where tumor cells express vimentin uniformly, and there are rare, but detectable T cells in the tumor stroma (blue arrows). (D) Samples from DOS-116, where tumor cells express vimentin uniformly, and there are detectable macrophages (black arrow) and neutrophils (yellow arrowhead) only within and at the boundary of areas of necrosis.

We also had the opportunity to analyze tumor and normal whole exome sequencing data for 88 dogs with osteosarcoma, and specifically to assess other potential mechanisms that might explain the patterns of immune cell infiltration in osteosarcoma. The exomic landscape of canine osteosarcoma has been previously described. One study was restricted tumors from three breeds¹⁹, which could have biased the results if specific variants or mutations (single nucleotide polymorphisms or substitutions or copy number variants or alterations) were enriched in breeds. The second study had a more diverse representation of breeds²⁰, but reached essentially the same conclusions. Our characterization of the genomic landscape of canine osteosarcoma provides yet another potential mechanism to explain the variation in immune and inflammatory infiltrates in osteosarcoma, as well as the potential difference in the relationship between immune infiltration and outcome in canine and human osteosarcoma. This work is part of a manuscript that is being prepared for publication.

Paired tumor normal exome seq capture allows identification of somatic mutations and copy number changes in tumor tissue. We combined new samples from our sequencing effort with the 59 samples from Sakthikumar et al¹⁹ in our analysis. Somatic mutations were identified utilizing a pipeline we designed to call mutations at specific locations based on the signal to noise ratios at each given position allowing a common analyses method across both human and canine tumor/normal samples. Somatic mutations were observed in every tumor in both species and, as reported previously in the Sakthikumar and the Gardner studies, non-synonymous mutations in *TP53* were highly recurrent in canine osteosarcoma (**Figure 11A**). *TP53* was also the most commonly mutated gene in human osteosarcoma (**Figure 11B**). These results are highly consistent with the known biology of osteosarcoma where *TP53* mutation has been described as an early event in OS tumor formation. In addition, we generated RNASeq expression data from 31 tumors, from three cell lines generated from these tumors, and from normal canine osteoblasts (the DOS dataset). Examination of the *TP53* mutations in the RNASEQ data validated that these mutations were directly observable in the transcriptional data and were also maintained in the cell lines generated from these tumors. While additional events were recurrently observed in a low percentage of both human and canine samples, the majority of these are associated with very large genes (like *SETD2*) which have been observed to be mutated in many tumors, and which do not have irrefutable oncogenic functions. These results lead us to conclude that the genomic landscape of osteosarcoma is chaotic in both species, and that somatic mutations do not explain the differential phenotypes of osteosarcoma observed in human and canine samples.

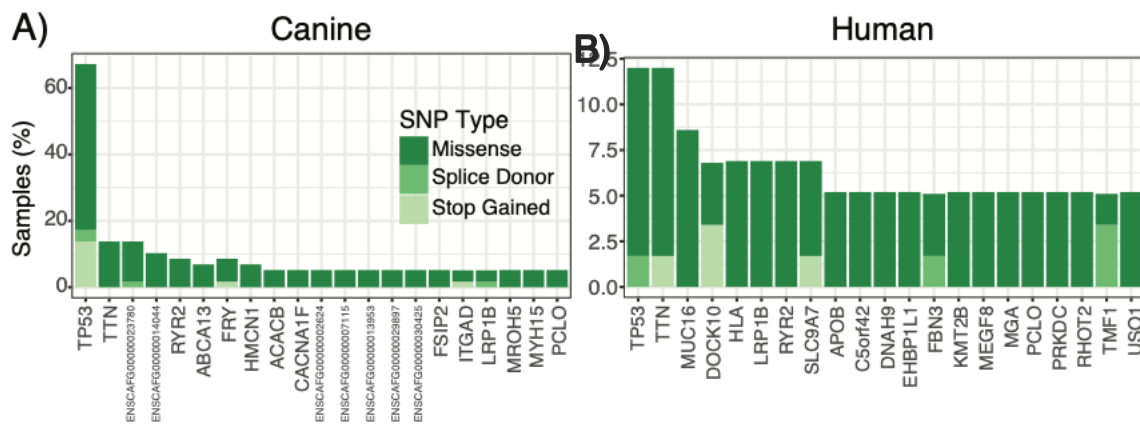


Figure 11. Frequency of single nucleotide polymorphisms in canine and human osteosarcoma. Analysis of tumor and normal (T:N) whole exome sequencing (WES) in canine human and osteosarcoma. The *TP53* gene showed the most frequent single nucleotide variants (single base pair mutations) in tumor, compared to normal for both canine (A) and human (B) samples. The overall rate of tumors with *TP53* inactivation, including those where there are copy number variants or other silencing mechanisms is approximately 70-75% in both species, but the dog seems to be preferentially susceptible to single base pair substitutions as a mechanism for activation. There was also a modest rate (approx. 5-15%) of single base pair substitutions in the *TTN*, *RPR2*, and *PCLO* genes in both species.

Mechanistic evidence for the events responsible for somatic mutation can be inferred by the types and neighbor-based environment of somatic mutation deviation from expected rates (*i.e.*, “COSMIC signatures”). Different tumor types show different patterns, for example skin cancers show UV light damage and lung cancers show enrichment for smoking related damage patterns. The signatures of these processes can be extracted from somatic mutations and a model can fit

the data to look at their relative impact. We used the COSMIC terminology to describe the mutational processes in human and canine osteosarcoma: osteosarcoma tumors from both species showed shared mutational processes as defined by the presence of COSMIC signatures 1 and 3.

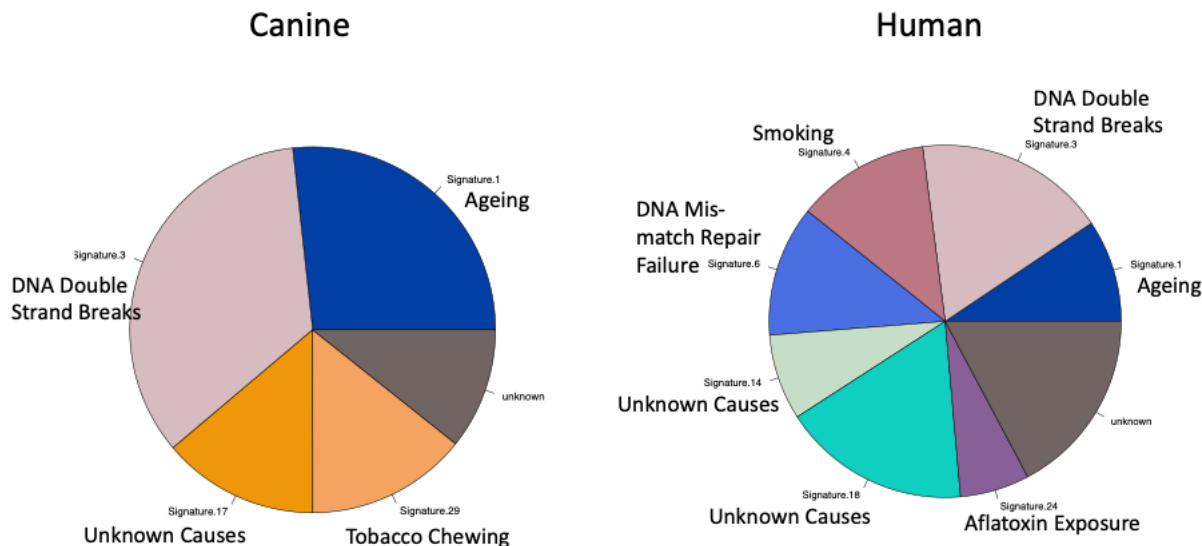


Figure 12. COSMIC signatures in canine and human osteosarcoma. The trinucleotide context of somatic single nucleotide variants was evaluated to assess mutation signatures across canine and human osteosarcoma samples using a Bayesian non-negative matrix factorization method. COSMIC signatures 1 and 3 were observed in tumors from both species, but while they accounted for approx. 65% of the contextual single base pair substitutions in canine osteosarcoma, they only accounted about 25% of mutations in human osteosarcoma. Overall, the mutational context in canine osteosarcoma showed less heterogeneity than what was observed in human osteosarcoma. A consistent pattern that would explain the etiology of these tumors, separate from the risks associated with cell division and aging, was not observed in either species.

COSMIC signature 1, which is characterized by C > T substitutions within the CpG trinucleotide context and is due to spontaneous deamination of 5-methylcytosine, likely secondary to aging (number of cellular replications), was more common in canine osteosarcoma than in human osteosarcoma (**Figure 12**). The most common signature identified in the canine dataset, however, was COSMIC signature 3, which is associated with failure of DNA double-strand break-repair by homologous recombination, and specifically with elevated numbers of large (longer than 3bp) insertions and deletions with overlapping microhomology at breakpoint junctions. COSMIC signature 3 was also more common in canine osteosarcoma than in human osteosarcoma (**Figure 12**). The remaining COSMIC signatures in tumors from dogs and humans were largely independent, suggesting that the etiology of human and canine osteosarcoma is different, although the tumors develop convergent cellular and morphological organization and transcriptional programs, which ultimately leads to comparable biological behavior.

Mutations in the *TP53* pathway allow for the clonal generation of somatic copy number aberrations which are widespread and canonically observed in both human and canine osteosarcoma. Copy number changes in canonical cancer-associated genes like *MYC* and *RB1* have been reported to be driver/progression events in osteosarcoma. To examine copy number changes in osteosarcoma, we summarized the tumor normal reads obtained from the sequence capture to window segments and looked at the copy number changes within these windows

(**Figure 13**). Expected copy number changes were observed on chrX and chrY. These serve as internal controls to show what loss of a single copy would look like (chrX) and also what complete loss of a region would look like (chrY). High level copy number gains were observed to be present in *MYC* and *RUNX2* in both species. We were also able to observe micro-deletions in regions containing *CDKN2A*, *RBI*, *PTEN* and *TP53* corresponding to loss of either one or both copies of the chromosomal region. Overall, the data show that canine osteosarcomas are less sensitive to amplification (copy number gain) than human osteosarcomas, while they are generally as susceptible, or more, to copy number losses.

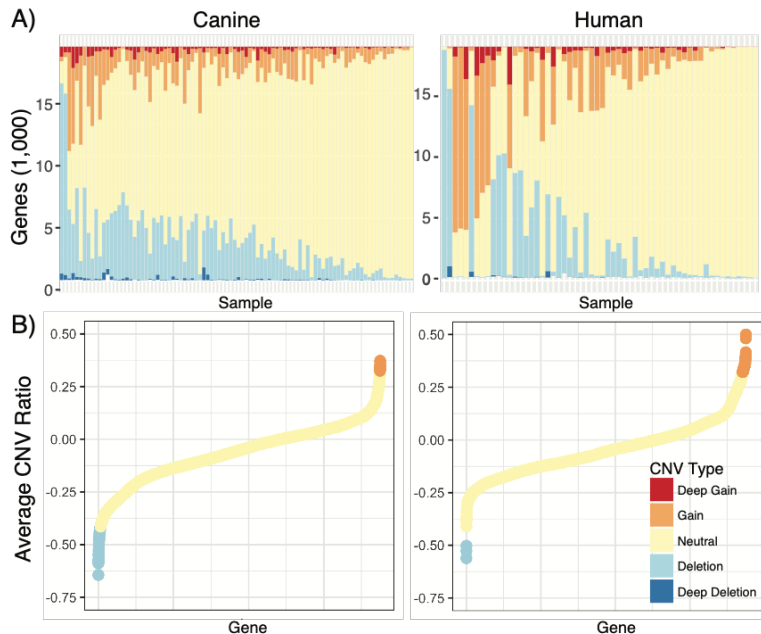


Figure 13. Copy number variation in canine and human osteosarcoma. (A) Copy number variants identified in WES of canine and human osteosarcomas are represented for each sample. The proportion of genes that are stable in the genome (neutral copy number variation) are represented in yellow. Copy number gains are represented by orange shading and deep copy number gains are represented by red shading. Copy number losses (deletion) are represented by light blue shading and deep copy number losses are represented by dark blue shading. Samples are ordered from least to most stable (based on copy number variation) from left to right. The data show that canine osteosarcomas are less sensitive to amplification (copy number gain) than human osteosarcomas, while they are generally as susceptible, or more, to copy number losses. (B) Copy number variation is represented for individual genes in canine osteosarcoma and human osteosarcoma. The data recapitulate the greater frequency of deleted genes in canine osteosarcoma and the greater frequency of amplified genes in human osteosarcoma.

To directly compare copy number changes across species we can simply sum the differences in tumor vs. normal for each gene and then divide by the number of tumors. This gives a single value which represents how often a specific gene is lost or gained within a tumor dataset (**Figure 14**). Although known targets like *MYC* and *RUNX2* are present in the extreme ends of these list, so are all of the genes in their chromosomal proximity potentially obscuring key tumor driver events. We identified the *PTEN* locus as the most commonly lost region in canine osteosarcoma. This was remarkable because *PTEN* loss is extremely rare in human osteosarcoma. Further, we observed loss of *PTEN* in many different breeds, indicating that homozygous loss of *PTEN* is not a breed specific event. Interestingly, the *PTEN* break point does not appear to be tightly conserved, and in many cases both copies are lost via different breakpoints. Specific examples also exist where an internal deletion in *PTEN* is observed maintaining the region of chromosome 26 distal to *PTEN*. It looks like genome instability as a result of *TP53* mutation leads to chromosomal breaks, and the most commonly observed critical (putatively causal) event that follows in the development of osteosarcoma is loss of both copies of *PTEN*. In the dog, *PTEN* is part of a small ~10-gene synteny block that has been transferred to the distal end of chromosome 26. This rearrangement goes back at least 3-4 million years to the ancestor of the grey wolf. In all other species we have examined, *PTEN* is contained within a larger synteny block in a much

more central chromosomal location. This location of *PTEN* in the dog genome seems to make it particularly vulnerable to deletion, and especially in the unstable genomic environment created by inactivation of *TP53* in osteosarcoma, which ultimately results in patterns approaching the clinical definition of chromothripsis.

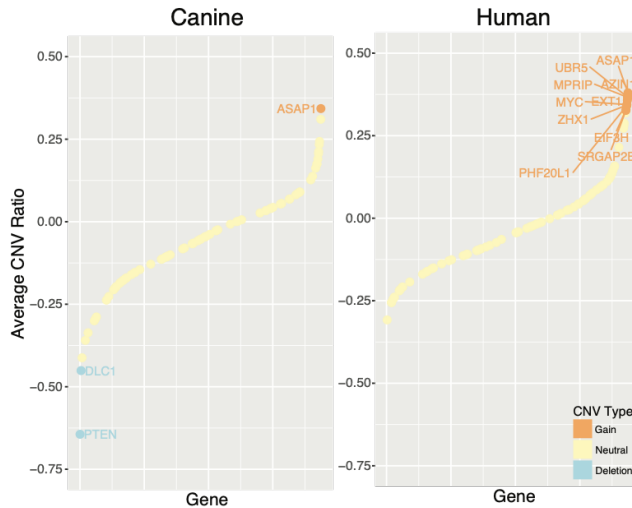


Figure 14. Homozygous loss of *PTEN* is exclusively observed in canine osteosarcoma. The representation of copy number gains and losses (Y-axis) is shown as a function of individual genes (X-axis) for canine osteosarcoma and human osteosarcoma, respectively. Human tumors show recurrent copy number gains in a number of genes, including *MYC*, *ASAP1* (also observed in the dog), and others, while homozygous deletion of *PTEN* is only observed as a recurrent event in canine osteosarcoma.

PTEN encodes a bifunctional protein and lipid phosphatase that acts as a central regulator of the phosphoinositide-3 kinase (PI3K) and Akt pathway that regulates cellular metabolism, growth, and survival. *PTEN* copy number in canine osteosarcomas was strongly correlated with *PTEN* transcript abundance (**Figure 15**). It is worth noting that *PTEN* transcripts were undetectable in two cell lines with homozygous deletion of this gene, whereas *PTEN* was robustly expressed in normal canine osteoblasts and in one cell line that retained both copies of the gene. This creates an intriguing dichotomy when compared to human osteosarcomas, as the overall levels of *PTEN* expression in canine osteosarcomas are variable, while the majority of human tumors show relatively low levels of *PTEN* (**Figure 16**). This is in spite of the relatively stable *PTEN* copy number in human osteosarcomas, suggesting that alternative mechanisms of *PTEN* regulation are at play in the human tumors.

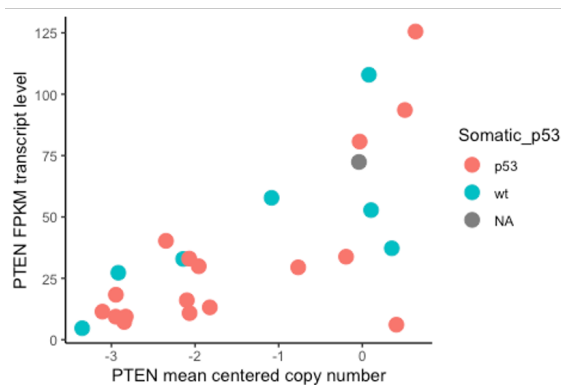


Figure 15. Correlation between *PTEN* copy number and *PTEN* transcript abundance in canine osteosarcoma. *PTEN* transcript abundance, determined from RNA sequencing as fragments per kilobase per million reads (FPKM) is shown as a function of *PTEN* copy number for canine osteosarcoma samples. Samples with wild type p53 (the product of the *TP53* gene) are shaded green, while samples with mutant p53 are shaded red. One sample where the sequence of p53 was indeterminate is shaded grey. The correlation coefficient is 0.71 for *PTEN* transcript abundance and copy number in this canine osteosarcoma cohort.

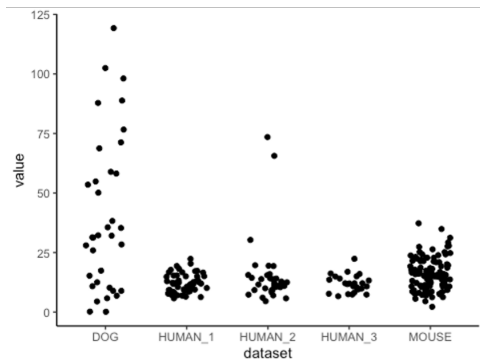


Figure 16. *PTEN* transcript abundance in canine, human, and mouse osteosarcoma samples. *PTEN* transcript abundance, determined from RNA sequencing as fragments per kilobase per million reads (FPKM) is shown for canine osteosarcoma samples, three unique cohorts of human osteosarcoma samples, and mouse osteosarcoma samples (generated through transposon-mediated mutagenesis). Note the heterogeneity of *PTEN* expression (transcript abundance) in canine osteosarcoma as compared to human and mouse osteosarcoma, where *PTEN* expression is strongly suppressed.

Based on the functional role of *PTEN*, we predicted that *PTEN* copy number and *PTEN* transcript abundance would be correlated with the cell cycle GCESS in dogs. Our results confirmed that hypothesis: both homozygous loss of *PTEN* and reduced *PTEN* transcript levels were associated with significantly increased cell cycle GCESS (**Figure 17**). Consistent with this observation, dogs with tumors that had homozygous deletions of *PTEN* showed significantly worse outcomes than dogs with tumors that had replete *PTEN*. Therefore, we can propose a unique model for osteosarcoma development in dogs, which starts with disruption of p53 function, most often due to loss-of-function mutations in either the *TP53* gene itself or in *CDKN2A*. The main risk factors for these mutations in dogs appear to be the number of replications in osteoblast progenitor cells combined with aging. Loss of p53 function, in turn, leads to error-prone replication of an unstable genome, predisposing for the loss of *PTEN* due to its vulnerable location at the distal arm of chromosome 26. Combined, the inactivation of p53 and the loss of Pten allow for the accumulation of additional mutations and tumor formation. As noted above, this finding also the conclusion that different risk factors and different molecular mechanisms are responsible for the etiology of human and canine osteosarcoma. However, once formed, the tumors develop convergent cellular and morphological organization and transcriptional programs, which ultimately leads to comparable biological behavior^{1,21-23}.

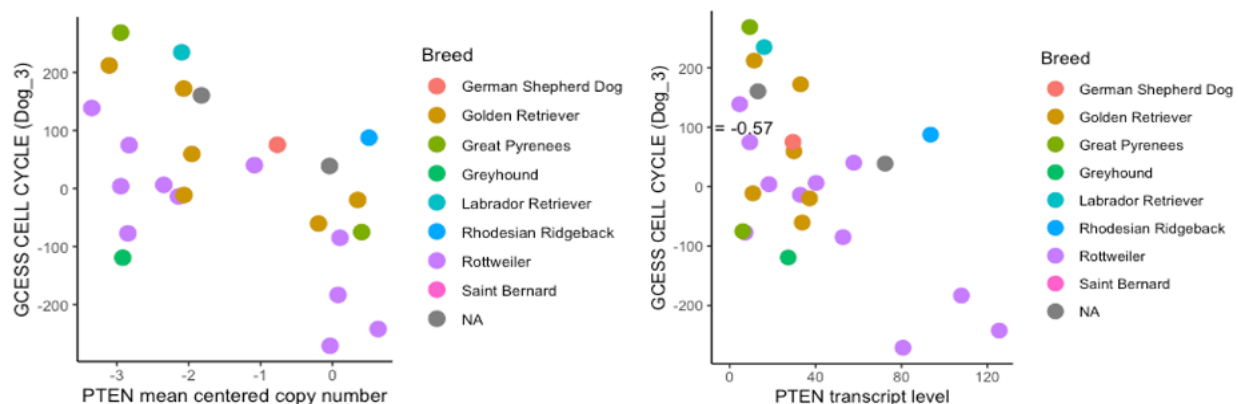


Figure 17. Correlation of *PTEN* copy number and *PTEN* transcript abundance with cell cycle GCESS in canine osteosarcoma. Gene cluster expression summary scores (GCESS) for cell cycle in canine osteosarcoma samples (Y-axis) are shown as a function of *PTEN* copy number (left panel) and *PTEN* transcript abundance (right panel). Dog breeds are illustrated by the color of each circle, where each circle represents an individual sample. Note the relatively higher cell cycle GCESS in samples with homozygous deletion of *PTEN*, and the accentuation of this division when the analysis is done using transcript levels.

One of the most surprising aspects of the mutational landscape of osteosarcoma was its relatively low total mutational burden, estimated at approx. 1.98 mutations per Mb²⁰, even within the observed genomic complexity and the presence of extensive translocations and fusion genes^{20,24}. The genomic complexity, however, would be predicted to generate a relatively large number of neoantigens, creating a fertile environment for expansion of tumor-specific T cells. This could be exaggerated in dogs, where the combined loss of function of p53 and Pten would be predicted to generate even more chaotic genomes. And yet, the total number of T cells in canine osteosarcomas are relatively low, as shown in **Figure 10**. This finding was corroborated when we examined the total number of CD8α transcripts in osteosarcoma (**Figure 18**).

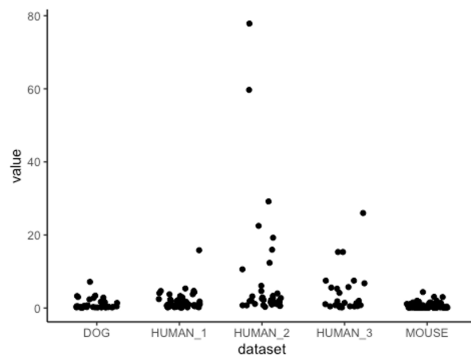


Figure 18. CD8α transcript abundance in canine, human, and mouse osteosarcoma samples. CD8α transcript abundance, determined from RNA sequencing as fragments per kilobase per million reads (FPKM) is shown for canine osteosarcoma samples, three unique cohorts of human osteosarcoma samples, and mouse osteosarcoma samples (generated through transposon-mediated mutagenesis). Note the relatively low content of CD8α, indicative of very few (or no) cytotoxic T cells present in the samples with only few outliers detectable in two of the three human sample cohorts.

This result, showing homogeneous low abundance of CD8α transcripts in dogs with the presence of outliers that showed higher abundance (albeit still relatively low expression) of CD8α transcripts in humans was somewhat surprising when compared to the estimates obtained by methylome sequencing, which indicated that canine osteosarcomas would have greater abundance of CD8 T cells than human osteosarcomas. The discrepancy might be the result of an indirect inference (methylation patterns) vs. a direct measurement (CD8α transcripts), but it could also result from low transcriptional activity of the CD8α gene itself, as the protein is relatively stable and the steady state levels of CD8α mRNA might be kept at relatively low levels.

As we noted before, *PTEN* transcript abundance was variable in the dog, so the suppression of CD8α transcripts was not likely to be due to poor quality of sequence or other artifacts. Nevertheless, we examined expression of the *MKI67* gene, which encodes the proliferation marker Ki-67 that is part of the cell cycle GCESS. **Figure 19** shows that each of the dog, human, and mouse osteosarcoma sample cohorts contained the expected variation of *MKI67* transcript abundance, consistent with the observed heterogeneity we previously reported in the cell cycle GCESS¹.

Finally, we asked whether there *PTEN* copy number and *PTEN* transcript abundance would be inversely correlated with the immune GCESS in dogs. Based on the data presented above, we hypothesized that loss of function of p53 and Pten would generate more neoantigens and attract more T cells. To our surprise, the data showed just the opposite. Copy number variation and

expression of *PTEN* were directly correlated with immune infiltrates, as determined by the immune GCESS (**Figure 20**).

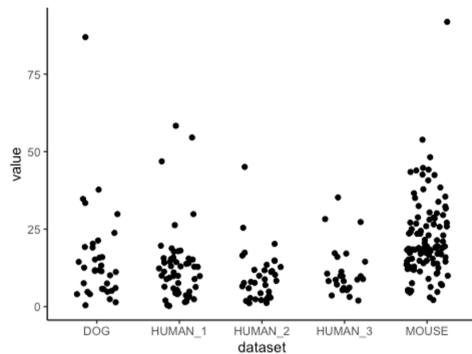


Figure 19. *MKI67* transcript abundance in canine, human, and mouse osteosarcoma samples. *MKI67* transcript abundance, determined from RNA sequencing as fragments per kilobase per million reads (FPKM) is shown for canine osteosarcoma samples, three unique cohorts of human osteosarcoma samples, and mouse osteosarcoma samples (generated through transposon-mediated mutagenesis). Note the expected variance in *MKI67* transcript levels (as predicted from the cell cycle GCESS) in each of the sample cohorts tested.

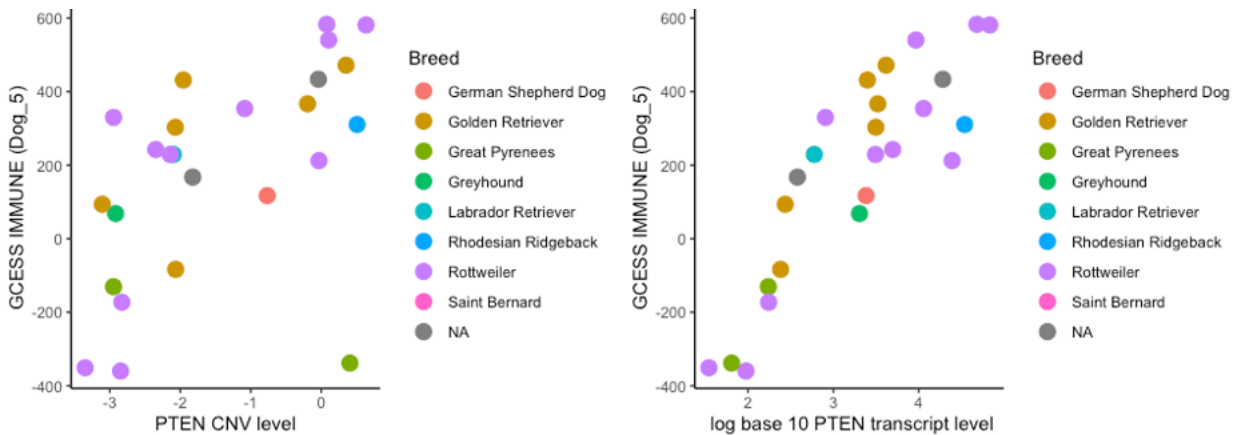


Figure 20. Correlation of *PTEN* copy number and *PTEN* transcript abundance with immune GCESS in canine osteosarcoma. Gene cluster expression summary scores (GCESS) for the immune cluster in canine osteosarcoma samples (Y-axis) is shown as a function of *PTEN* copy number (left panel) and *PTEN* transcript abundance (transformed to log values, right panel). Dog breeds are illustrated by the color of each circle, where each circle represents an individual sample. Note the relatively higher immune GCESS in samples that retain *PTEN*, and the accentuated relationship when the analysis is done using log-transformed transcript levels.

We continue to study the foundation for these unexpected results, although they highlight one potentially important difference that must be taken into account when extrapolating data from canine osteosarcoma studies to humans. Additional work will be needed to establish potential differences in the composition of immune infiltrates, as well as to define the mechanisms through which T cells are excluded from the stromal environment of osteosarcomas. It is apparent that treatments which break this barrier for T cell entry can generate long-term responses that result in prolonged survival, and perhaps even cures in dogs with osteosarcoma¹³, and a major focus should be on developing such therapies in a manner that can be readily translated to routine clinical use in both dogs and in humans.

Specific Aim 1, Task 5: Prepare and sequence exosomes from canine osteosarcoma cell lines and their paired osteosarcoma tissues of origin.

For this task, we showed that the cargo in exosomes derived from cell lines and the cargo derived from tumor cells in an *in vivo* context are very different, creating limitations to the applicability of *in vitro* models to understand exosome packaging and the distant effects of exosomes.

Specific Aim 1, Task 5, Subtask 1: For this specific experiment, we will compare exosome cargo from 20 osteosarcoma cell lines. Exosomes will be purified from cell lines using established methods.

To extend the rationale for these experiments, we examined whether exosome production by osteosarcoma cells was positively correlated with tumor progression. These experiments were done as part of a collaboration with Dr. Brad Bryan who, at the time, was at Texas Tech University Health Sciences Center in El Paso, TX. The experiments were completed prior to the initiation of this project²⁵, but their analysis and integration into a systematic and comprehensive assessment of the role of exosomes to mold the osteosarcoma environment were done through effort supported by this project.

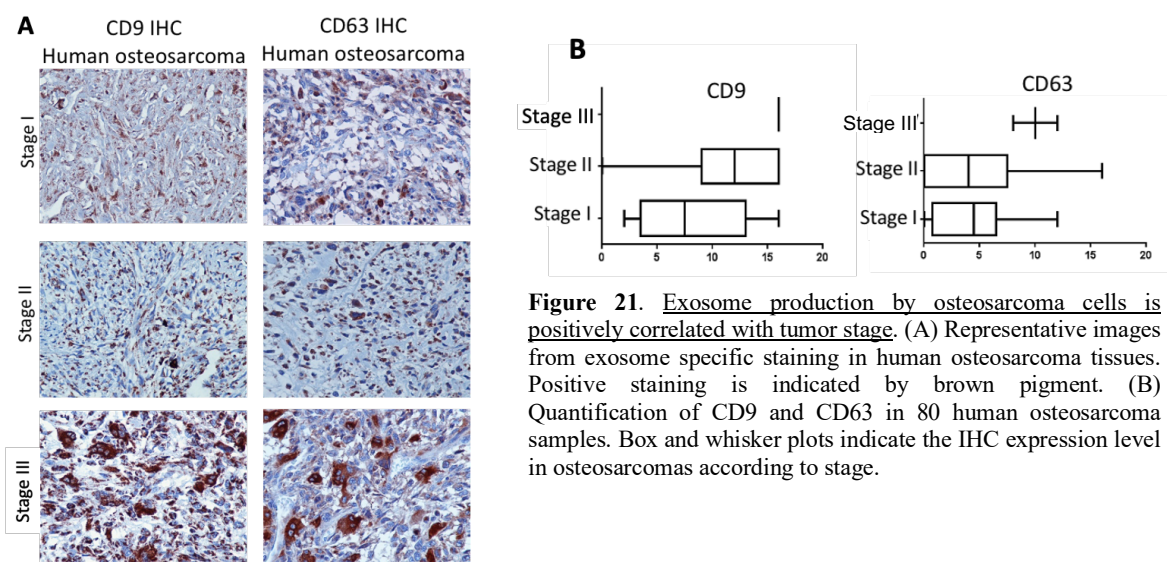


Figure 21. Exosome production by osteosarcoma cells is positively correlated with tumor stage. (A) Representative images from exosome specific staining in human osteosarcoma tissues. Positive staining is indicated by brown pigment. (B) Quantification of CD9 and CD63 in 80 human osteosarcoma samples. Box and whisker plots indicate the IHC expression level in osteosarcomas according to stage.

Previous studies showed a direct relationship between behavior aggressiveness and the level of exosomes produced in various tumor types, but this relationship has not been conclusively established for sarcomas, and particularly for osteosarcomas. To investigate this in the context of osteosarcoma, specifically, the presence of tetraspanins CD9 and CD63, both of which are enriched in exosomes, was quantified in 80 human osteosarcoma samples using immunohistochemistry (IHC) (**Figure 21**). The data show that stage-III tumors stained more robustly for both CD9 and CD63 than stage-I or stage-II tumors, suggesting that a positive relationship between total detectable exosomes and tumor stage also exists in osteosarcoma. To further delineate the functional involvement of exosomes in the progression of osteosarcoma, we continued our experiments in the more tractable canine model system. We validated exosome production by canine osteosarcoma cell lines using immunofluorescence. **Figure 22** shows positive staining for CD81, CD63 and CD9 in secreted microvesicles from OSCA-40 and

OSCA-32 canine osteosarcoma cell lines, indicating conservation of tetraspanins in extracellular vesicles -most likely representing exosomes- from humans and dogs.

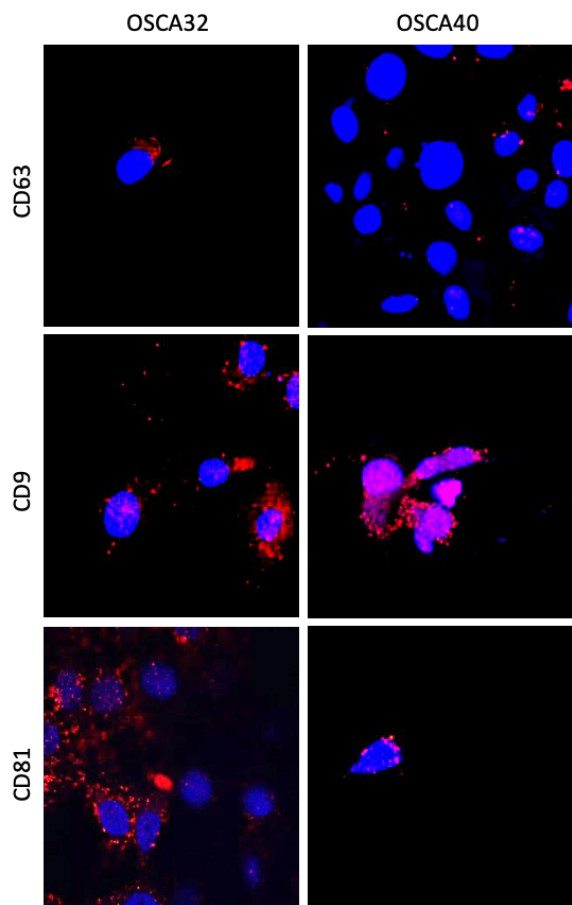


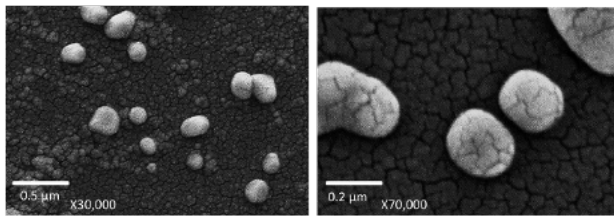
Figure 22. Tetraspanins are detectable in microvesicles secreted by canine osteosarcoma cell lines. Cultured dog osteosarcoma cell lines, OSCA-32 and OSCA-40, were stained for tetraspanins CD63, CD9, and CD81 and imaged using immunofluorescence.

To confirm the physical properties of canine osteosarcoma-derived extracellular vesicles as exosomes and to validate their enrichment, we utilized scanning electron microscopy (SEM), NanoSight particle tracking analysis, and immunoblotting. These experiments were also part of our collaboration with Dr. Bryan, as detailed above. SEM showed spherical microvesicles between 100 and 200 nm in diameter; this size and shape was consistent with that predicted for exosomes (**Figure 23A**). Additionally, immunoblotting showed enrichment of CD63 and depletion of β -actin in the exosomes relative to the whole cell lysates (**Figure 23B**). Finally, nanoparticle tracking analysis showed that the mean vesicle size ranged from 149 nm to 180 nm with a mode of 117 nm to 132 nm, (**Figure 23C**). This range is similar to the size determined by SEM and is consistent with the expected size of exosomes.

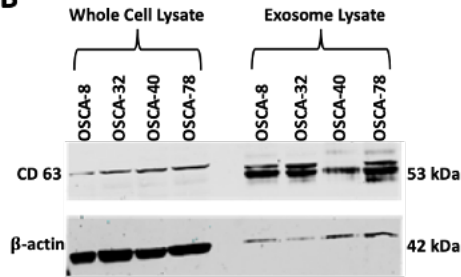
We next confirmed that exosomes enriched from serum samples of a dog with osteosarcoma and a dog with no evidence of disease were comparable in size to cell line-derived exosomes (**Figure 24**). In all, these findings support the methodology used for exosome enrichment from cell lines and from serum samples.

We next sequenced exosomes from normal dermal canine fibroblasts, normal canine osteoblasts, and osteosarcoma cells from dogs, humans, and mice. For these experiments, cells were cultured for 48 hours in exosome-free media, at which time the supernatant was collected, and exosomes were isolated using a modified protocol for the ExoQuick TC kit (SBI). Exosomes were quantified using the NanoSight particle tracking system. The results show that the exosomes prepared from canine, human, and mouse osteosarcoma cell lines had similar physical properties (**Figure 25**).

A



B



C

Nanoparticle Size Analysis of Isolated Exosomes

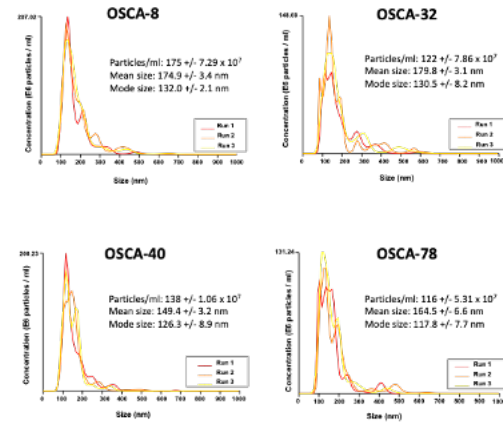


Figure 23. Physical and biochemical characterization of canine osteosarcoma-derived exosomes. (A) Scanning electron micrographs of OSCA-40 exosomes (B) Immunoblotting of exosome preparations documenting enrichment of tetraspanins (CD63) and depletion of β -actin in OSCA-8, OSCA-32, OSCA-40, and OSCA-78 dog osteosarcoma cell lines. (C) NanoSight particle tracking analysis of triplicate samples each of osteosarcoma cell lines OSCA-8, OSCA-32, OSCA-40, and OSCA-78 showing modal diameters of approximately 117-132 nm.

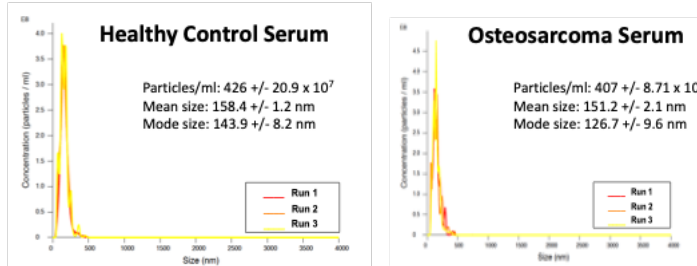
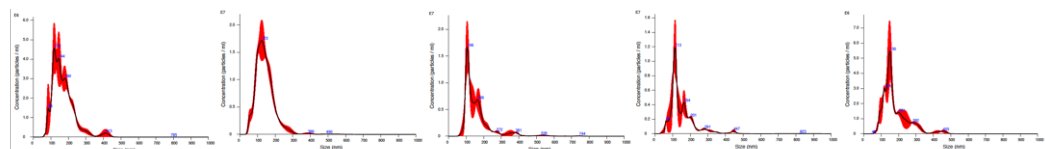


Figure 24. Characterization of canine serum derived exosomes NanoSight particle tracking analysis of triplicate samples of serum from healthy dog and a dog with osteosarcoma. Note the X-axis scale is different than in Figure 23, but the modal diameter of the exosomes remains in the range of 126-144 nm.



Cell Type	Normal Canine Fibroblasts	Normal Canine Osteoblasts	Canine Osteosarcoma	Human Osteosarcoma	Mouse Osteosarcoma
Mean Particle Diameter in nm ($\pm$ SEM)	169 (7.7)	142 (4.6)	161 (1.6)	152 (5.1)	180 (5.8)
Particle Concentration/mL ($\pm$ S.D.)	4.95e+8 (2.74e+7)	1.86e+9 (1.77e+8)	1.10e+9 (3.19e+7)	7.303+8 (4.083+7)	4.383+8 (9.26e+7)

Figure 25. NanoSight tracings for exosomes derived from cultured canine fibroblasts, canine osteoblasts, and representative canine (OSCA-40), human (Saos-2), and mouse (K12) osteosarcoma cell lines. Mean particle diameter in nm ($\pm$ SEM) and the particle concentration (per ml) are tabulated in the bottom panel.

Specific Aim 1, Task 5, Subtask 2: RNA isolation from exosomes.

Please see the narrative under Subtask 3 below for details.

Specific Aim 1, Task 5, Subtask 3: Nanoscale library preparation and next generation sequencing will be done at the University of Minnesota Genomics Center using conventional methods.

To determine whether exosomes contents were reflective of cytosolic content, and whether exosomes from cell lines were conserved with exosome contents secreted by tumors *in vivo*, we sequenced mRNA from two canine cell lines and from exosomes derived from the same cell lines under conventional culture conditions. At the same time, we sequenced tumor xenografts and exosomes from the harboring xenografts of the same cells. We then used a bioinformatics pipeline to establish sequences of dog origin in the mouse serum-derived exosomes. The data from the cultured cell lines and from the xenograft tumors has been previously reported^{1,6}. For this project, we focused on analysis of data from the exosomes. We found poor correlations between cellular mRNAs and exosomal mRNAs, indicating that mRNAs are actively loaded into exosomes and they are not simply a reflection of cellular mRNA content or a window to ascertain cellular mRNA abundance. Previous experiments done in our lab also indicated that exosomal microRNAs were not reflective of cellular microRNA content. Moreover, the exosomal transcripts identified in cultured canine osteosarcoma cells showed only minimal overlap (1.4%) with the exosomal transcripts derived from the same canine cell lines when they formed tumors *in vivo*, suggesting that the microenvironment is a major factor influencing exosome loading. These results tempered our enthusiasm to extrapolate how exosomes derived from *in vitro* cultured cell lines might influence immune cells *in vitro*, providing a major reason to support our emphasis on sequencing data from tumors over *in vitro* experiments. Even so, we completed critical experiments in Specific Aim 2 using cell culture models to address the hypotheses offered in the original proposal.

Specific Aim 1, Task 6: Define statistical correlations of exosome microRNAs and immune transcript abundance in tumors and lymph nodes.

This task was deferred due to technical obstacles generating exosomes from preserved, frozen tissues and the likely futility of performing the analyses in cultured cells.

Specific Aim 1, Task 6, Subtask 1: Next generation RNA sequencing for mRNA and small RNAs will be completed for three paired tumor samples, cell lines, exosomes, and lymph node mRNAs at the University of Minnesota Genomics Core using conventional methods.

Please see the narrative for Specific Aim 1, Task 4 and Task 5 above and Subtask 2 below for details. As noted above, the main objective for the lymph node samples obtained as part of the oncolytic virus clinical study was to examine lymphocyte clonality in dogs after treatment with an oncolytic virus, so RNA sequencing of lymph node samples was not done.

Specific Aim 1, Task 6, Subtask 2: Statistical correlations between microRNA clusters identified in exosomes and gene clusters defined in tumor tissues, in cell lines, and in lymph nodes, will be done as described in Task 4.

Unfortunately, the ExoQuick method was unsuitable to enrich exosomes from frozen samples, as it does not have a means to exclude cellular debris from lysed cells. Most other available exosome enrichment methods, including ultracentrifugation had the same problem, and so we were unable to establish reliable methods to extract exosomes from frozen tumor tissues prior to the pandemic. We identified a kit that might achieve reliable exosome enrichment from frozen tissues (the Capture EM kit) late in the summer of 2020. However, because of limitations on lab work during the pandemic, we were unable to test whether this method would provide suitable preparations to complete this subtask. As noted above, we de-prioritized analysis from *in vitro* cultures and we did not perform RNA sequencing from lymph nodes.

Specific Aim 1, Task 7: Build tissue microarrays.

A canine osteosarcoma tissue microarray was built. The COVID-19 pandemic delayed completion of the material so that only quality control was able to be done. However, the resource will be available for future investigations.

Specific Aim 1, Task 7, Subtask 1: We have obtained approximately 400 samples from 100 dogs with osteosarcoma from the CCOGC biorepository, which will provide another independent dataset for validation.

H&E slides were made and evaluated for every case available.

Specific Aim 1, Task 7, Subtask 2: Tissue microarrays (TMAs) will be built by selecting well-preserved blocks of tissue that have been consistently processed, and for which suitable tissue and stroma are available based on their H&E staining characteristics. Each block will be annotated to obtain 4 distinct cores, representing tumor and stroma. Sixty-four cores per block will be spotted randomly to create a new tissue microarray of naïve (untreated) canine osteosarcoma samples (projected n=48).

Thirty-six samples were deemed to be suitable for inclusion in the TMA. Slides were marked and the tissues went into the queue to build the TMA in December of 2019. Maps were designed in January 2020; however, the TMA was not built by the time the University ceased operations in March of 2020 in response to the shelter-in-place mandates to combat the spread of COVID-19. The Bionet histology lab reopened in August of 2020 and the TMA is now built and undergoing QC (H&E and vimentin IHC). Unfortunately, the TMA was not completed in time to perform the staining experiments.

Specific Aim 1, Task 7, Subtask 3: Quality assurance and quality control will be done by cutting and staining serial samples with H&E and anti-vimentin IHC (V9 antibody). Cores in each block will be evaluated for completeness, folds, and consistent staining.

See Subtask 2 above. QC is ongoing, but the TMA was not completed in a timely manner due to the closures required by the COVID-19 pandemic.

Specific Aim 1, Task 8: Immunohistochemistry.

Immunohistochemical analysis of osteosarcoma samples had been done previously, but as noted above, generation of the tissue microarray was delayed, and so confirmation of previous immunohistochemistry data (replication) could not be done before the end of the project.

Specific Aim 1, Task 8, Subtask 1: The presence of CD3⁺ and CD28⁺ T cells, as well as antigen presenting cells expressing the CD28 ligands, CD80 and CD86, PAX5 B-cells, and Mac387⁺ macrophages and granulocytes in tumor tissues will be evaluated semi-quantitatively in TMA slides by at least 2 independent pathologists.

We validated immunostaining in canine osteosarcoma samples for CD3, PAX5, and MAC387 (see **Figure 10**). We identified antibodies for validation against canine CD28, CD80, and CD86. However, we were unable to complete these experiments due to closures in response to the COVID-19 pandemic (see above).

Specific Aim 2: To establish mechanisms of CD28 silencing by osteosarcoma-derived exosomes.

The hypothesis under test for this aim was that CD28 expression in T cells within the tumor environment was downregulated by tumor-derived extracellular vesicles (TEVs or exosomes). This hypothesis was developed based on work by Dr. Subramanian's lab in colorectal cancer. Dr. Subramanian was a co-investigator on this project and is an internationally recognized authority on microRNA biology.

Specifically, Dr. Subramanian's group showed that TEVs containing microRNA miR-424(miR-322 in mice) suppressed the CD28-CD80/86 costimulatory pathway in tumor infiltrating T cells and dendritic cells in a syngeneic mouse model of colorectal cancer, leading to resistance against immune checkpoint blockade therapies. Modified TEVs with miR-424 knocked down enhanced T-cell mediated antitumor immune response in this colorectal cancer model and increased the response of immune checkpoint blockade therapy. Intravenous injections of modified TEVs also induced tumor antigen specific immune responses. **The original submitted version of this manuscript authored by Dr. Subramanian, which is currently under revision for publication, is included as an appendix to this report.**

Specific Aim 2, Task 1: Cell line authentication (human, dog, mouse) and Mycoplasma screening.

This task was done on a quarterly basis, or as frequently as needed for cells that were maintained in culture for prolonged passages per our lab SOPs.

Specific Aim 2, Task 2: Generate Dicer knockout osteosarcoma cell lines and controls along with stable microRNA knockdown cell lines and controls.

Cells with knockdowns of CD28-targeting microRNAs were generated for colorectal cancer models. But given our data showing no correlation between CD28-targeting microRNAs and

immune regulation in osteosarcoma, we deferred generation of Dicer knockout osteosarcoma cell lines and microRNA knockout osteosarcoma cell lines for this project.

Specific Aim 2, Task 2, Subtask 1: Cells will be edited using resources available at the Masonic Cancer Center Genome Editing Shared Resource.

This task was initially delayed due to the departure of two of our project technicians (see section 5) and we had proposed to reduce the scope to generate one mouse and one human cell line for experiments. miR-424(miR-322) knockouts were established for the CT-26 cell line (see appendix). However, before we embarked on this expensive endeavor, we used the resources we already had at our disposal to determine if the experiments were likely to be informative.

Given the lack of correlation between microRNAs and immune transcripts in Specific Aim 1, we performed an initial correlation analysis of expression of microRNAs that target canine CD28 mRNA, and CD28 transcript abundance to establish the potential for microRNA targeting for this critical T-cell molecule. Our initial assessment suggested that the canine miR-424 orthologue was anti-correlated with CD28 transcript levels in canine osteosarcomas (**Table 5**). We thus decided to evaluate these relationships systematically in our two independent canine datasets.

Table 5. Correlation between microRNAs predicted to target canine CD28 and CD28 mRNA levels in canine osteosarcoma samples.

MiRNA_name	Correlation Estimate	P value
cfa-miR-146a	0.6107	0.0001
cfa-miR-146b	0.0486	0.7881
cfa-miR-15a	0.1105	0.5405
cfa-miR-15b	-0.3246	0.0653
cfa-miR-183	-0.5238	0.0017
cfa-miR-202	0.2414	0.1759
cfa-miR-25	-0.1509	0.4018
cfa-miR-27a	0.0496	0.7838
cfa-miR-27b	-0.1113	0.5374
cfa-miR-31	-0.2739	0.1229
cfa-miR-424	-0.4347	0.0114
cfa-miR-503	0.0929	0.6073

First, we evaluated whether specific immune transcripts, such as CD28, moved independently from the immune GCESS (specifically, the macrophage and the T-cell cluster scores), which would have suggested that this gene was regulated independently from other transcripts in the infiltrating immune cells. In other words, we would have expected to see T cell transcripts, such as CD2, CD3, CD4/CD8, CD25, Tbet, Lck, etc. clustered together in some or many of the samples, but perhaps not the CD28 co-receptor or its ligands CD80 and CD86.

Figure 26 shows unsupervised clustering for the two independent canine osteosarcoma sequencing datasets (DOS and CCB), with gene expression clusters highlighted on the right axis.

The cell cycle cluster and the two independent immune clusters are identified by color coding (red, green, purple). The quality of sequencing of the new CCB dataset allowed for inclusion of greater number of genes in the analysis. The data show that CD28 was consistently present as part of the T-cell cluster, and its relative abundance was consistent with other T-cell related genes in all the samples.

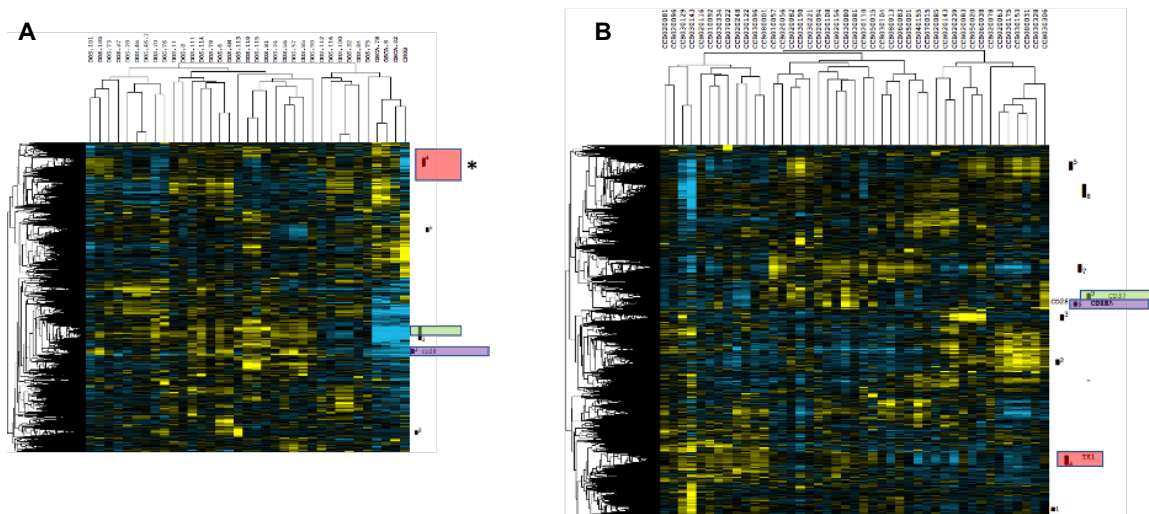


Figure 26. Identification of gene expression clusters in two independent datasets of dogs with osteosarcoma. The two independent canine osteosarcoma cells sequenced are shown. The DOS dataset is shown in panel A and the CCB dataset is shown in panel B. The two datasets were quantile normalized, $\log_2$ transformed, and mean centered. Hierarchical clustering was done using average linkage with Pearson correlation. Gene clusters were derived to include 60 or more genes with a direct correlation of 0.6. The cell cycle cluster is denoted by red boxes in the right axis for each diagram, the immune macrophage cluster is denoted by green boxes, and the T-cell cluster is denoted by purple boxes. CD28 was invariably within the T-cell cluster in every sample.

These analyses were based on identification of co-regulated gene clusters and had not considered individual molecules, for example, CD3, CD8, CD28, etc. to be significant in that context, since single biomarkers are notoriously unreliable to establish presence or absence of biological molecules. Nonetheless, in spite of the apparent co-regulation of CD28 with the rest of the detectable immune transcripts, we considered that the evidence of direct targeting of CD28 in colorectal tumors (see appendix) was sufficient to compel further examination of the hypothesis that CD28 was a direct target of tumor-derived microRNAs in osteosarcoma.

We used the data from human and canine osteosarcoma samples for which we had completed mRNA sequencing and microRNA sequencing to assess expression of CD28 in the samples (an indicator of T-cells that were present in the sample), as well as the abundance of CD28-targeting microRNAs; that is, any microRNA predicted to bind the 3' untranslated region (UTR) of full length human or canine CD28 (**Figure 27**). For our initial experiment, each microRNA (total CPMs) was plotted independently against the transcript counts for CD28 (FPKMs) for its corresponding tumor for human samples (A) and canine samples (B). Blue lines show linear regression for the two variables as a measure of correlation. A sum of the total microRNAs in A and B is shown in C and D (human and dog, respectively) to assess correlation of transcript abundance for CD28 and microRNAs that target its 3'UTR region. Note the different scales, indicating more abundant CD28 mRNA and more abundant CD28-targeting microRNAs in the

canine samples, compared with a relative dearth of both CD28 mRNA and CD28-targeting microRNAs in the human samples.

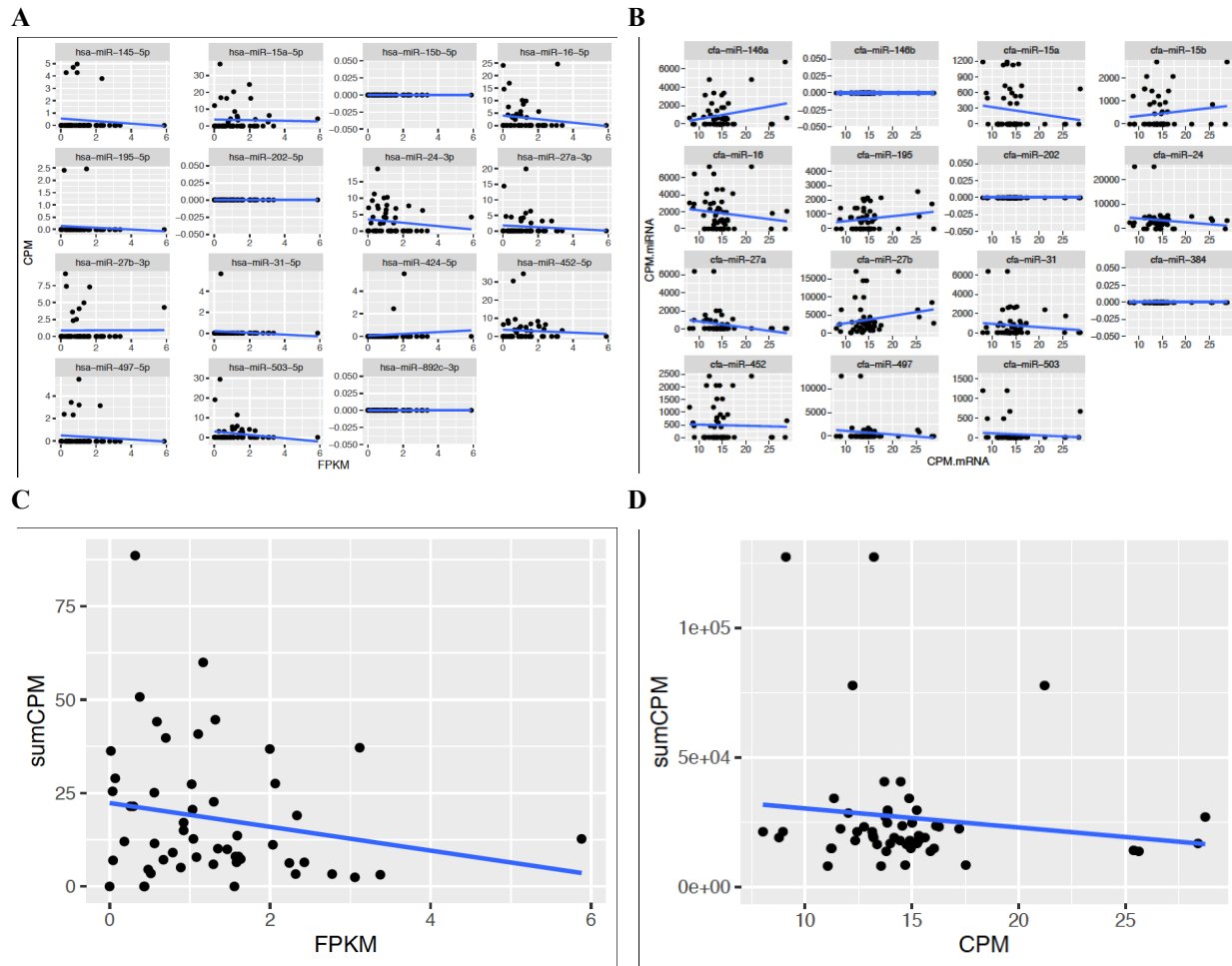


Figure 27. Correlations between abundance of microRNAs targeting the CD28 3'UTR and CD28 transcripts in human and canine osteosarcoma. MicroRNAs were selected for analysis that are reported to target the CD28 3'UTR (hsa-miR-145-5p, hsa-miR-15a-5p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-195-5p, hsa-miR-202-5p, hsa-miR-24-3p, hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-31-5p, hsa-miR-424-5p, hsa-miR-497-5p, hsa-miR-503-5p, and hsa-miR-829c-3p in humans and cfa-miR-146a, cfa-miR-146b, cfa-miR-15a, cfa-miR-15b, cfa-miR-16, cfa-miR-195, cfa-miR-202, cfa-miR-24, cfa-miR-27a, cfa-miR-27b, cfa-miR-31, cfa-miR-384, cfa-miR-452, cfa-miR-497, and cfa-miR-503 in dogs). Each microRNA (total CPMs) was plotted independently against the transcript counts for CD28 (FPKM) for its corresponding tumor for human samples (A) and canine samples (B). Blue lines show linear regression for the two variables as a measure of correlation. A sum of the total microRNAs in A and B is shown in C and D (human and dog, respectively) to assess correlation of transcript abundance for CD28 and microRNAs that target its 3'UTR region. Note the different scales, indicating abundant CD28-targeting microRNAs in canine samples, compared with a relative dearth of CD28-targeting microRNAs in human samples.

The presence of immune infiltrates in dogs and humans was one of the most highly conserved interactions in osteosarcoma from both species. However, even as expression of CD28 itself was somewhat different, it was quite low in the osteosarcoma samples from both humans and dogs, near the lower limit of detection (**Figure 27**). These low expression levels of CD28 are consistent with the data shown for CD8a in **Figure 18** and suggest that the overall presence of T cells in osteosarcoma tumor tissues from both species is generally quite low (see also **Figure 10**). One intriguing difference noted above is that CD28-targeting microRNAs were expressed at

vanishingly low levels in humans, while they were present at higher levels in dogs - albeit with a fairly large degree of heterogeneity (**Figure 27**). One possible interpretation of these data is that the mechanisms leading to downregulation of CD28 might be different in humans and in dogs. For example, the events leading to downregulation of CD28 in humans, as well as other effects to alter anti-tumor T cell responses, might occur in distant tissues, such as the draining lymph node, whereas these immunoregulatory steps might occur within the tumor in dogs .

Our next experiment was to test whether these findings would replicate in an independent dataset. For this, we used the most CCB canine osteosarcoma dataset and compared the results with the DOS canine osteosarcoma dataset. The first step was to establish whether the overall expression of microRNAs in the two datasets were comparable. The results show that, even with the improved mapping of sequences from the most recent dataset, the trends for expression of individual mRNAs of interest were similar (**Figure 28**). Note that miR-424 was excluded from the analysis, as the total counts for this microRNA were extremely low, raising some doubt about the robustness of the data from the initial analyses shown in **Table 5**.

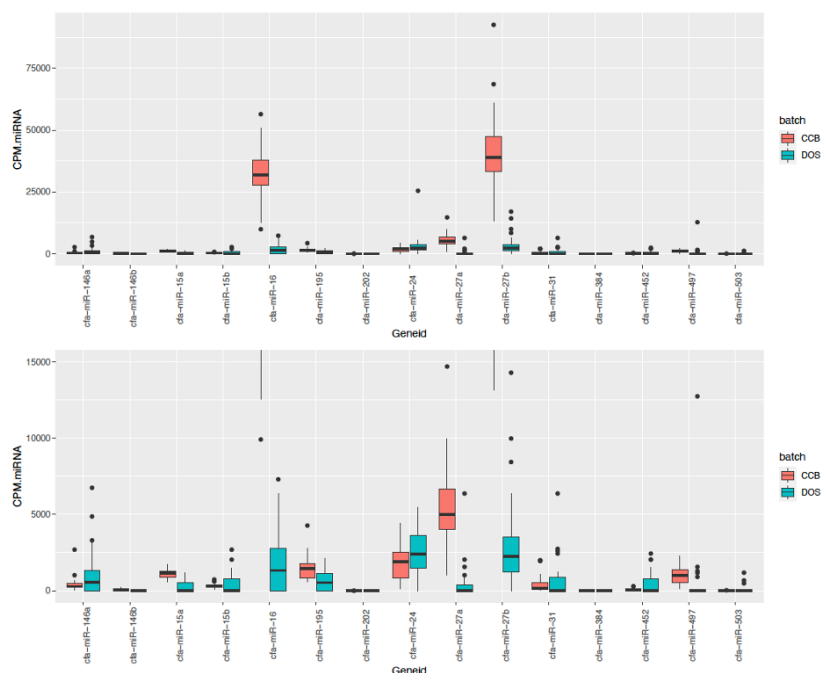


Figure 28. Comparative expression of selected microRNAs targeting canine CD28 in two independent next generation sequencing datasets of canine osteosarcoma. Analysis of normalized counts per million (CPM) for 15 microRNAs that are predicted to target the 5' UTR of canine CD28. The top panel shows the full scale of CPMs on the Y-axis. The bottom panel is a zoomed-in version to compare the microRNAs with lower expression between the two datasets. DOS represents the original dataset and CCB represents the most recently sequenced dataset.

Figure 29 shows that the trends of anti-correlation between canine microRNAs and canine CD28 mRNA in the DOS dataset did not replicate in the CCB dataset, suggesting the original relationships from the DOS dataset might have been spurious. One important difference between these two datasets was the overall counts for CD28 mRNA (see the X-axis scale in **Figure 29**). The CCB dataset was in line with what we observed in the human dataset, with regard to total T-cell abundance (as measured by CD28 counts), whereas the DOS dataset included higher CD28 counts. The potential for greater numbers of infiltrating T cells might also have made the

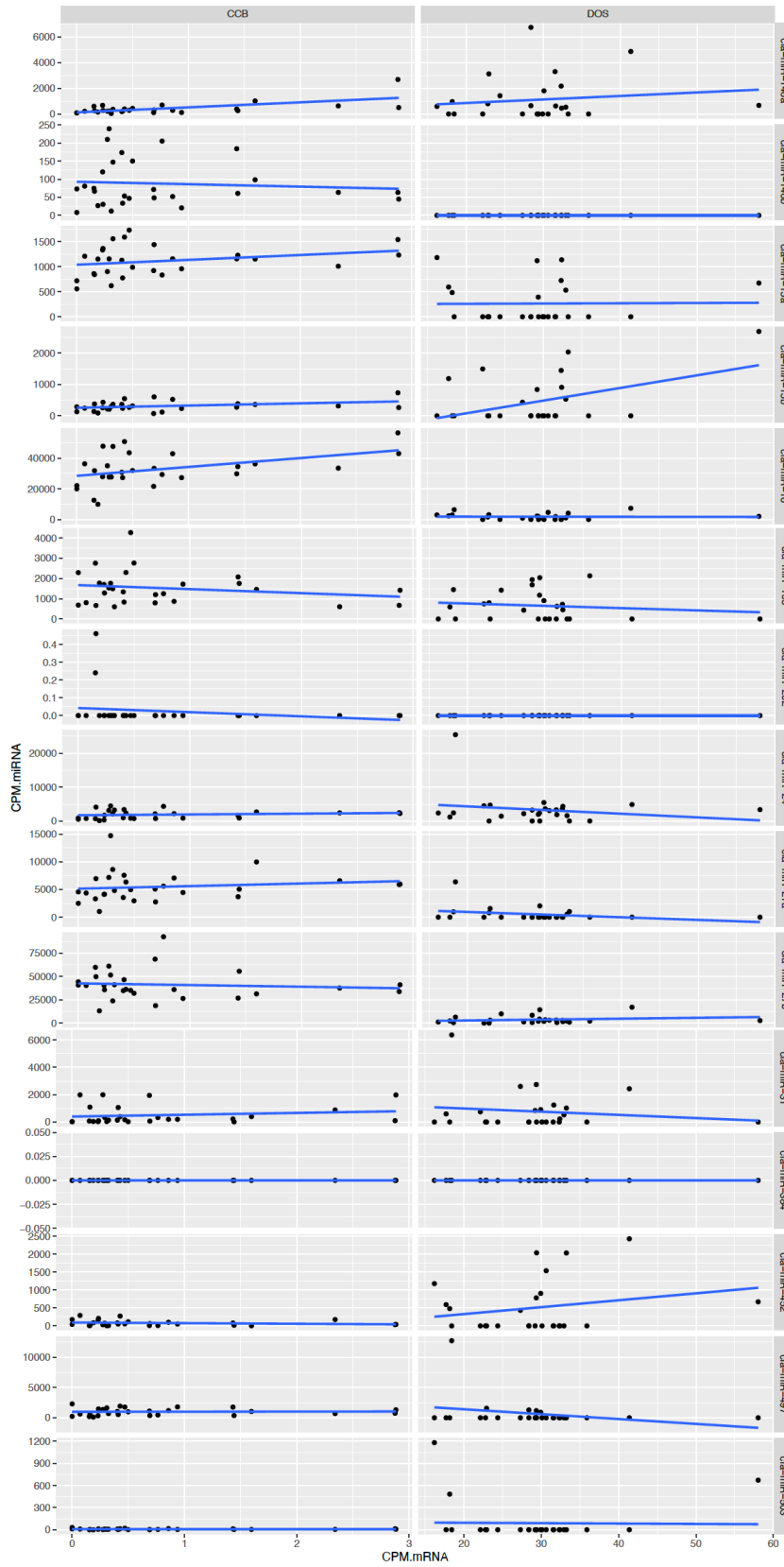


Figure 29. Comparison of correlations between abundance of microRNAs targeting the CD28 3'UTR and CD28 transcripts in two independent datasets of canine osteosarcoma. The left panels show expression of microRNAs (CPM in the Y-axis) as a function of expression of CD28 mRNA (CPM in the X-axis) for the CCB dataset. The right panels show expression of microRNAs (CPM in the Y-axis) as a function of expression of CD28 mRNA (CPM in the X-axis) for the DOS dataset. In order from top to bottom, the microRNAs included were cfa-miR-146a, cfa-miR-146b, cfa-miR-15a, cfa-miR-15b, cfa-miR-16, cfa-miR-195, cfa-miR-202, cfa-miR-24, cfa-miR-27a, cfa-miR-27b, cfa-miR-31, cfa-miR-384, cfa-miR-452, cfa-miR-497, and cfa-miR-503. Blue lines show linear regression for the two variables as a measure of correlation. Note the variable Y-axis scales, indicating significant differences in abundance of CD28-targeting microRNAs (see also **Figure 28**), and the scales in the X-axis showing

relationships easier to observe, but consistent with the predictions from the GCESS analysis, more than 85% of the samples were below the median in both datasets, as they were in the

human samples. We believe this diminishes the likelihood that the CCB data are outliers, and we thus concluded that this observation documented a general exclusion of T-cells from the osteosarcoma environment that was unlikely to be mediated exclusively by exosomal microRNA-mediated suppression of CD28. Given the disparate observations in osteosarcoma and colorectal cancer, we also conclude that CD28 targeting by exosomal microRNAs occurs in some cancer types, but it is not a universal mechanism of tumor-mediated immunosuppression.

Following that conclusion, we chose not to proceed with the generation of DICER knockdown osteosarcoma cells for Task 2, subtask 1.

Specific Aim 2, Task 3: Obtain T cells from apheresis residues (human), peripheral blood (dogs), or spleens (mice).

Cells were obtained and stored for experiments.

Specific Aim 2, Task 3, Subtask 1: Human PBMCs will be obtained from approved commercial sources.

Human apheresis residues were obtained from Memorial Blood Center in Minneapolis, MN. The human Jurkat T-cell line is a commonly used model of T-cell activation derived from a human leukemia which retains intact antigen receptor-dependent pathways of activation that promote IL-2 production. Jurkat cells were obtained from the ATCC and were stored frozen for experiments in our laboratory. Authentication was completed through IDEXX BioResearch, according to our lab SOPs as described for cell lines above.

Specific Aim 2, Task 3, Subtask 2: Canine PBMCs will be prepared from whole blood obtained from health pet dogs with owner consent.

Canine blood samples were obtained from the Veterinary Medical Center (Clinical Investigation Center) in St. Paul, MN.

Specific Aim 2, Task 3, Subtask 3: mouse T cells will be prepared from spleens and lymph nodes obtained from normal donor.

Given the data for human and canine cells (see below), we did not sacrifice mice for this subtask, as the experiments were considered to be futile. This is consistent with our efforts to eliminate unnecessary use of animals for research.

Specific Aim 2, Task 4: Isolate exosomes from tumor cells and apply to species-matched T cells to quantify exosome uptake (fluorescence) and examine expression of CD28 and other genes.

Exosomes were purified from cells, characterized, and stored for experiments. The results of our experiments show that exosomes could alter behavior of stromal cells in the metastatic niche, but they generally did not affect expression of CD28 and only had modest effects on T cell activation.

Specific Aim 2, Task 4, Subtask 1: We will confirm release of microRNAs from exosomes

using RT-PCR with species-mismatched T cells.

Upon further reflection for the design of this experiment, we concluded that differential RT-PCR would prove challenging to establish release of exosome contents in target cells, so we settled on a different approach. We transfected osteosarcoma cells with a CD81-green fluorescent protein (GFP) fusion protein and documented incorporation of the fusion protein into exosomes. Then, we overlayed enriched osteosarcoma exosomes onto species mis-matched target cells to assess uptake and alterations in cell function.

Given our inability to reject the null hypothesis; that is, our observations did not support a mechanism whereby exosomes established an immunosuppressive environment in osteosarcoma that was dependent on or mediated by repressing CD28 expression, we decided to use these experiments to also examine whether exosomes could alter the osteosarcoma niche. The experiments were done through our collaboration with Dr. Brad Bryan, then at the Texas Tech University Health Sciences Center in El Paso, TX.

The importance of exosomes in promoting a pre-metastatic niche has been characterized in several non-osteosarcoma cancers, such as pancreatic cancer and malignant melanoma. However, the ability of osteosarcoma exosomes to influence the complex cascade of events that occurs during metastasis, and particularly their impact on stromal cells within the microenvironment, has not been fully addressed. The importance of fibroblasts and endothelial cells in tumor progression have been well described, making them a useful model system to investigate exosome internalization and influence on cell behavior. Ascertainment that secreted tumor-derived exosomes can be taken up by stromal cells in the organ that is the major target of metastasis requires a method to track tumor specific exosomes. This was accomplished by transfecting canine osteosarcoma cells with CD81 linked to a GFP tag. Expression of the CD81-GFP fusion protein in transfected OSCA-40 cells, and its incorporation into secreted microvesicles, were visualized using immunofluorescence microscopy (**Figure 30**).

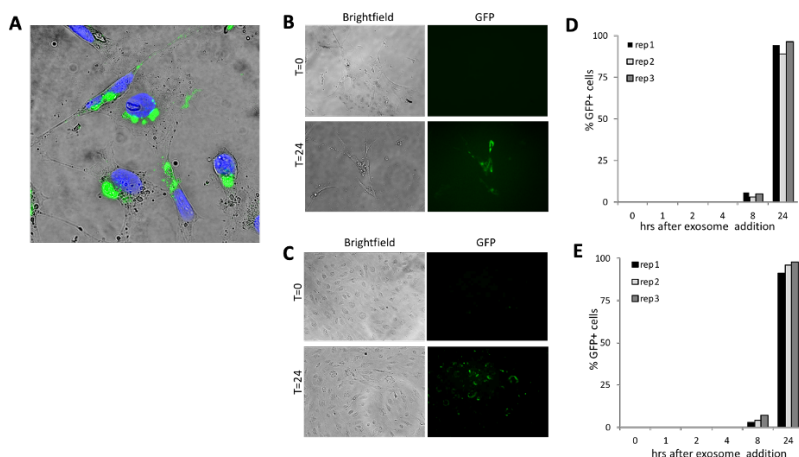


Figure 30. Osteosarcoma exosome are taken up by pulmonary fibroblasts and endothelial cells. (A) A CD81-GFP fusion vector was stably transfected into OSCA-40 canine osteosarcoma cells. Expression of the fusion protein (green) and its incorporation into secreted microvesicles was determined via immunofluorescent microscopy. Blue staining is the nucleus. (B & C) CD81-GFP tagged exosomes were purified from OSCA-40 cells and added to primary cultures of human pulmonary fibroblasts (B) or human pulmonary endothelial cells (C). Images of the GFP fluorescence were taken at 0, 1, 2, 4, 8, and 24 hours post treatment with the exosomes. (D & E) Histogram illustrating temporal changes in the number of GFP positive fibroblast and endothelial cells.

Lung stromal fibroblasts and lung endothelial cells were selected as the most relevant target cells for analysis. **Figures 30B, C, D, and E** show that GFP-positive osteosarcoma-derived exosomes

were internalized by human pulmonary fibroblasts or human pulmonary endothelial cells within 6 to 8 hours, with nearly 100% of the target cells showing GFP expression by 24 hours.

The effect of internalized exosomes on the behavior of stromal fibroblasts and endothelial cells was addressed using cell migration and proliferation assays. A scratch-filling assay was performed to determine the migration activity of human pulmonary fibroblasts and endothelial cells following addition of osteosarcoma exosomes. An increase in cell number as well as target cell migration following addition of exosomes was apparent, with a more pronounced effect on proliferation (**Figures 31A and B**). To address the underlying mechanisms for these changes, we used a bead array to interrogate how osteosarcoma-derived exosomes altered gene expression in human pulmonary fibroblasts. The use of canine exosomes, human target cells, and a human-specific bead array diminished the potential that we would detect canine mRNAs carried in the exosomes. The data show altered expression of genes mediating cell adhesion and motility, and inflammation (**Figure 31C**). These data underscore the notion that exosomes retain conserved mechanisms across species by demonstrating that canine-derived exosomes are readily taken up by human stromal cells. Furthermore, exosomes were shown to alter the behavior and gene expression of stromal cells consistent with events that are expected to occur within the tumor microenvironment during metastasis.

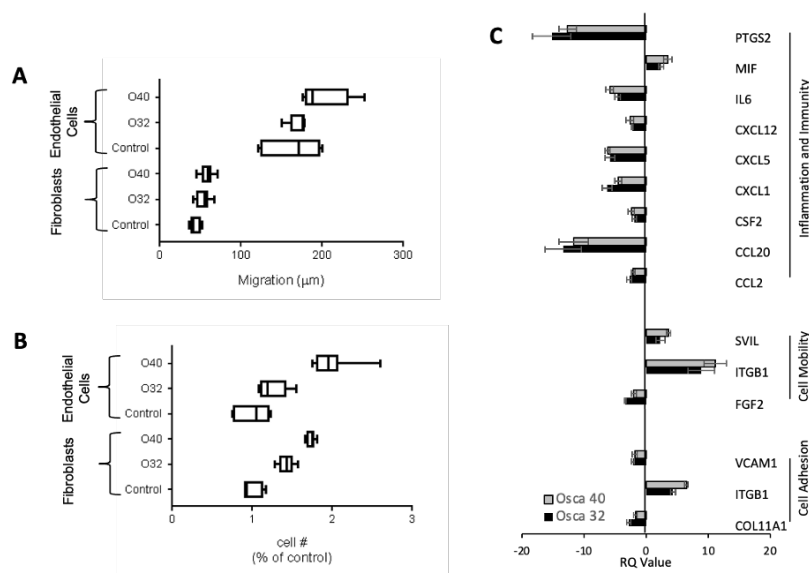


Figure 31. Osteosarcoma-derived exosomes alter behavior and gene expression of pulmonary fibroblasts and pulmonary endothelial cells. (A) Migration of pulmonary endothelial cells and pulmonary fibroblasts in a scratch-filling assay compared to control after exposure to osteosarcoma exosomes from OSCA-32 and OSCA-40 cell lines. (B) Proliferation of pulmonary endothelial cells and pulmonary fibroblasts compared to control after exposure to osteosarcoma exosomes from OSCA-32 or OSCA-40 cell lines. (C) Gene expression array analysis of human pulmonary fibroblast cells treated with exosomes from OSCA-32 or OSCA-40 cell lines. Data shown are genes whose expression changed relative to control by more than 2-fold.

Specific Aim 2, Task 4, Subtask 2: We will co-culture species-matched T cells and osteosarcoma-derived exosomes.

First, we examined whether CD28 was stable during T-cell activation, to ensure that any observed effect in the conditions where T cells were co-cultured with exosomes was not due to alterations induced as a consequence of T cell activation. As we reviewed the literature on CD28 stability, we were surprised to find sparse information on the subject. An interesting paper by Chris Rudd's group was the only study we could find evaluating the fate of CD28 in activated T cells²⁶. Rudd's group showed that after binding with anti-CD28 antibodies, as much as 35% of the surface protein is internalized and segregated into two pools. One pool was targeted for

degradation and the other pool was recycled. Despite small differences, the results from these experiments were similar in Jurkat cells and in primary human T cells. One intriguing observation was that CD28 molecules that were linked to the PI3K signaling complex were preferentially internalized and degraded, perhaps as a means to terminate signaling and allow cells to proceed on to implementation of their activation programs. Nonetheless, this means that even a major reduction of surface CD28 might still fail to affect T cell function in circumstances where the proportion linked to p85, and thus to PI3K activation, was still saturating. This portion might constitute as few as 15-20% of the total number of CD28 receptors in one T cell!

To test for stability of CD28 expression, we cultured human T cells without any added stimulation, or in the presence of PMA (10 nM) and ionomycin (500 nM) to induce activation for 18 hours. At the end of the culture period, cells were harvested, stained with antibodies against CD3, CD45, CD4, and CD28, and analyzed using flow cytometry. **Figure 32** shows predictable downregulation of CD3 and CD4 antigens from the cell surface, without significant modulation of CD45 and CD28 antigens off the cell surface. Our data showing a very modest reduction of CD28 are consistent with Rudd's data²⁶ documenting largely stable CD28 expression in T cells upon activation, with the change in mean fluorescence intensity being consistent with the estimates of about $\frac{1}{3}$ of the molecules being internalized and a proportion of those molecules likely recycling back to the cell surface.

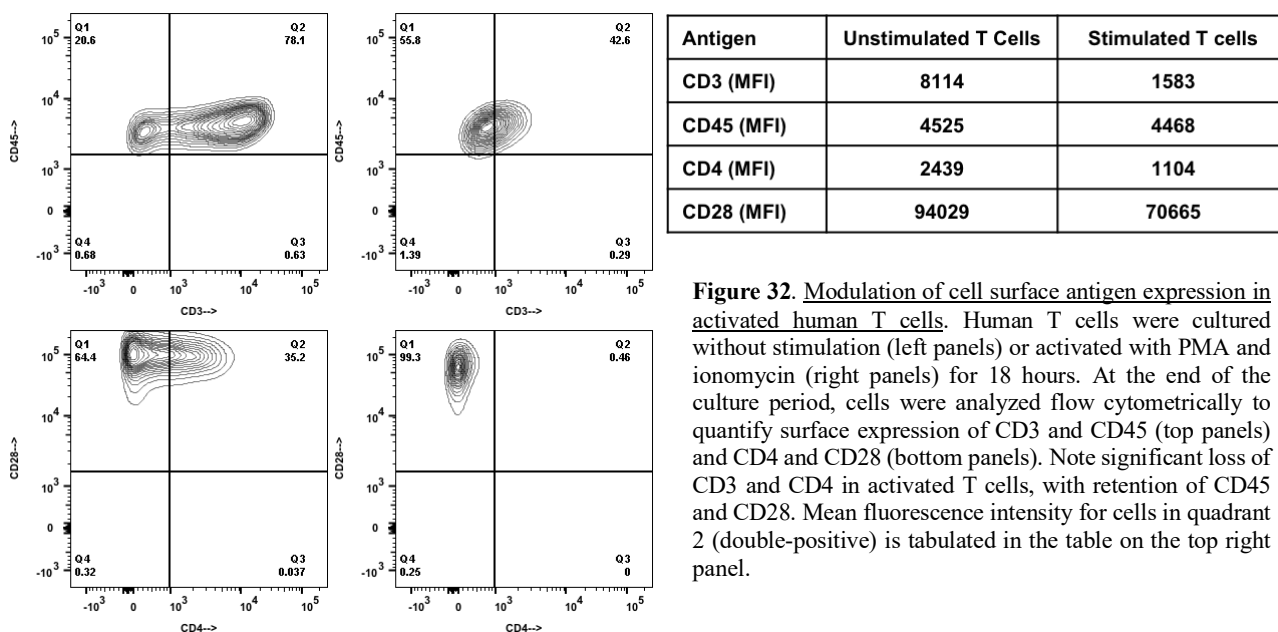


Figure 32. Modulation of cell surface antigen expression in activated human T cells. Human T cells were cultured without stimulation (left panels) or activated with PMA and ionomycin (right panels) for 18 hours. At the end of the culture period, cells were analyzed flow cytometrically to quantify surface expression of CD3 and CD45 (top panels) and CD4 and CD28 (bottom panels). Note significant loss of CD3 and CD4 in activated T cells, with retention of CD45 and CD28. Mean fluorescence intensity for cells in quadrant 2 (double-positive) is tabulated in the table on the top right panel.

We next examined whether exosomes would affect T-cell activation. Human T cells were stimulated with anti-CD3 and anti-CD28 antibodies conjugated to beads for 18 hours in exosome-free media with or without addition of exosomes from cultured osteosarcoma cells or control cells. Controls included normal canine dermal fibroblasts and normal canine osteoblasts. Osteosarcoma cells included three cell lines derived from canine tumors with different biological behavior (OSCA-40, most aggressive, OSCA-8, intermediate aggressiveness, OSCA-32, least aggressive) and a human osteosarcoma cell line (U2OS) that has less aggressive behavior. At the end of the culture period, supernatants were collected, and IL-2 production was quantified using

a commercial ELISA kit. **Figure 33** shows that human osteosarcoma-derived exosomes induced a dose-dependent reduction of IL-2 production by human T-cells, whereas no such reduction was observed with any of the canine-derived (osteosarcoma or normal cell) exosomes. This suggests that, unlike what we observed in pulmonary fibroblasts and endothelial cells, exposure of T cells to osteosarcoma-derived exosomes might impair their activation, but only if the exosomes are from the relevant species. This result was puzzling, but based on this, we continued the remainder of the experiments with species-matched exosomes.

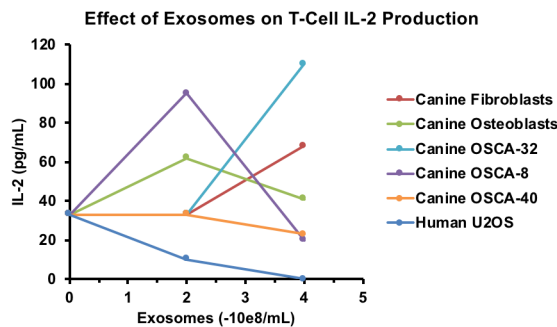


Figure 33. Effect of osteosarcoma-derived exosomes on human T-cell IL-2 production. Human T cells were stimulated with anti-CD3 + anti-CD28-conjugated beads cultured and in exosome-free medium for 18 hours with or without addition of exogenous osteosarcoma-derived or control cell exosomes. At the end of the culture period, IL-2 was quantified in supernatants using a commercial ELISA kit. Note absence of inhibition of IL-2 production in cultures that received canine cell-derived exosomes, and the dose-dependent inhibition of IL-2 production in T cells cultured with human osteosarcoma-derived exosomes.

Specific Aim 2, Task 4, Subtask 3: Osteosarcoma cells treated with amiloride to inhibit exosome production will be used to obtain material that will be used as a control for non-specific (non-exosome mediated) effects.

Since the previous results showed that only human osteosarcoma-derived, and not canine osteosarcoma-derived exosomes impaired IL-2 production by human T-cells, we concluded the amiloride experiments were not necessary.

Specific Aim 2, Task 4, Subtask 4: We will measure expression of CD28 and other immune response genes by T cells will be examined using flow cytometry and quantitative RT-PCR.

For these experiments, we used canine T cells, canine osteosarcoma-derived exosomes, and measured the effects on CD28 exclusively using flow cytometry. Considering the stability of the CD28 protein, as shown above, we did not believe that alterations in CD28 mRNA might have a meaningful effect if the levels of the CD28 protein on the surface were unaffected.

Canine T cells were cultured without stimulation or in the presence of lectin mitogens (concanavalin A, or ConA, or phytohemagglutinin, or PHA) for 24 and 48 hours, and the expression of CD28 was examined at the end of each culture interval. **Figure 34** and **Figure 35** show that CD28 expression in canine T cells was largely unaffected by the presence of species-matched osteosarcoma-derived exosomes. The concentration of exosomes used for these experiments exceeded the saturation level shown to inhibit IL-2 production (**Figure 33**), so we believe this was unlikely to be an effect of insufficient exosomes in the culture.

Specific Aim 2, Task 4, Subtask 5: We will measure T-cell proliferation using dye dilution assays, by flow cytometry, and T-cell activation using specific markers (CD25, CD69), also by flow cytometry.

We examined expression of CD25 in human and canine T cells cultured with osteosarcoma-derived exosomes and expression of CD69 in human cells. Consistent with the data shown above for CD28, we did not observe any effects from exosomes to reduce expression of these markers (data not shown). We also did not observe differences in viability or cell counts in these experiments, so we did not perform dye dilution experiments, as we believed such experiments were unnecessary in light of the negative data.

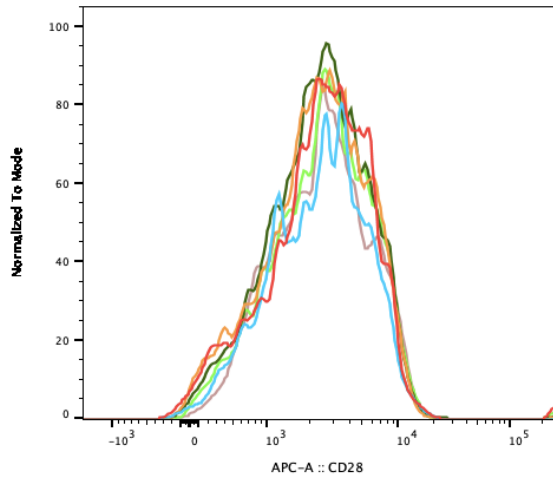


Figure 34. CD28 expression in canine T cells cultured with osteosarcoma-derived exosomes. Exosomes were enriched from the OSCA-32 and OSCA-40 canine osteosarcoma cell lines and added to canine PBMCs at a concentration of 1×10^9 exosomes/ml. Conditions tested included unstimulated T cells with no exosomes (red line), ConA-stimulated T cells with no exosomes (light blue line), unstimulated T cells with OSCA-32 exosomes (orange line), unstimulated T cells with OSCA-40 exosomes (dark blue line), ConA-stimulated T cells with OSCA-32 exosomes (green line), and ConA-stimulated T cells with OSCA-40 exosomes (brown line). Note the overlapping staining for every condition, indicating no alterations in CD28 expression in canine T cells cultured with canine osteosarcoma-derived exosomes.

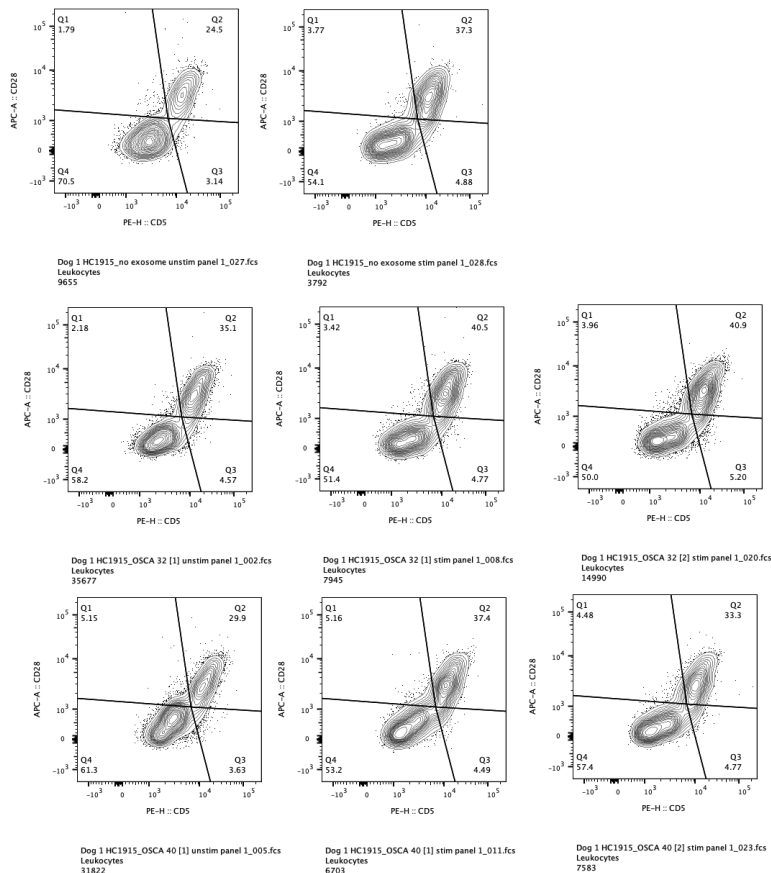


Figure 35. CD28 expression in canine T cells cultured with osteosarcoma-derived exosomes. Canine PBMCs were cultured with canine osteosarcoma-derived exosomes as described in **Figure 34**. The panels are 2-dimensional contour plots showing expression of CD28 in the Y-axis as a function of expression of CD5 (the prototypical marker for canine T cells) on the X-axis. Quadrants separate CD5/CD28-positive cells (T cells) from CD5/CD28-negative cells (non-T cells). Note the absence of CD5-positive and CD28-negative (or even CD28-dim) cells in any condition. The left-most panels show expression of CD5 and CD28 in unstimulated T cells. The middle panels and the right-most panels show expression of CD5 and CD28 in mitogen-stimulated T cells. The middle row shows data from PBMCs cultured with OSCA-32-derived exosomes and the bottom row shows data from PBMCs cultured with OSCA-40-derived exosomes. Similar results were obtained in experiments from at least 4 independent donor dogs.

Based on the data shown so far for specific aim-1 and specific aim-2, we continued our efforts on the project with a reduced emphasis on CD28 itself, and instead evaluating genome-wide relationships that could define the biology and the immunological landscape of osteosarcoma.

Specific Aim 2, Task 4, Subtask 6: We will examine T-cell migration towards chemokine gradients using Boyden chamber assays.

As noted above, our inability to reject the null hypothesis re-directed our efforts. It seems that the major immunosuppressive barrier in osteosarcoma is related to exclusion of immune cells. Our data create a possibility that this exclusion is mediated through reduced production of inflammatory and chemotactic cytokines by stromal cells (see **Figure 31C**). Considering the modest, yet counter effect of osteosarcoma-derived exosomes to promote migration of fibroblasts and endothelial cells in scratch wound assays, we concluded Boyden chamber assays to examine inhibition of T cell migration towards cytokine would probably not be informative, especially if the lesion occurs at the level of production of the cytokines themselves.

Specific Aim 3: To define the relationship between CD28 silencing and resistance to immune checkpoint blockade in pre-clinical osteosarcoma mouse models.

For this aim, we developed a novel molecule called P-DIC that interferes with binding of PD-1 with its PD-L1 and PD-L2 ligands as well as with binding of CD47 to its SIRP α ligand. This theoretically would block the myeloid checkpoint, enhancing tumor cell phagocytosis and antigen presentation, as well as the immune exhaustion checkpoint, enhancing tumor cell killing by cytotoxic lymphocytes and natural killer cells. We successfully synthesized the molecule, showed binding to the target receptors, and showed preliminary evidence of activity *in vitro* and *in vivo*. Completion of these experiments, however, was hindered by the COVID-19 lockdowns.

Specific Aim 3, Task 1: Production of in vivo grade high affinity stabilized sPD-1 variant to block PD-1/PD-L1 and PD-1/PD-L2 interactions.

As part of a collaboration with Dr. Aaron Ring, extending from his time as a graduate student at Stanford University and continuing through his initial faculty appointment at Yale University, we became aware of a novel molecule that he had designed but never synthesized or tested, henceforth referred to as P-DIC (peptide for dual immune checkpoint blockade) or P-DIC_{HI} (peptide for dual immune checkpoint inhibition). P-DIC incorporates a modified domain of SIRP γ , called gamma variant-3 that binds CD47 with orders-of-magnitude higher affinity than its natural ligand, SIRP α , joined by a 3-amino acid linker to a soluble PD-1 high affinity consensus (sPD1HAC) polypeptide that binds to PD-L1 and PD-L2 in in tumor cells and immunosuppressive macrophages with orders-of-magnitude higher affinity than the native PD-1 protein. The synthesis and production of P-DIC are described below.

Specific Aim 3, Task 1, Subtask 1: We will produce stabilized sPD-1 variant in *E. coli* (inclusion bodies).

The P-DIC sequence (see **Figure 38A**) was incorporated into *E. coli* and the bacterial cells were grown in a bioreactor in the University of Minnesota Molecular and Cellular Therapeutics facility. Dr. Daniel Vallera led the efforts to produce P-DIC using established methodology for

production of biological drugs under conditions that are suitable for future runs using good manufacturing practice (GMP) standards. The drug was purified from *E. coli* inclusion bodies through sequential column chromatography and re-folding that has been used successfully to produce other drugs in Dr. Vallera's drug development pipeline.

Specific Aim 3, Task 1, Subtask 2: We will produce stabilized sPD-1 variant using peptide synthesis (synthetic chemistry) by CRO.

Because P-DIC synthesis and production was successful in house using *E. coli* inclusion bodies, we did not need to seek synthesis of the product through a CRO.

Specific Aim 3, Task 2: Generation of genetically mouse osteosarcoma cells and controls using transposon systems (stable expression of CD81- or CD63-GFP, luciferase, OVA).

We generated a human cell line expressing CD63-GFP. Our efforts to generate mouse osteosarcoma cell lines expressing luciferase and OVA had limited success. We were able to complete a Material Transfer Agreement (MTA) with West Virginia University to obtain mouse osteosarcoma cells that express firefly luciferase (but not OVA), but negotiations over the MTA were delayed due to the COVID-19 pandemic and the document was signed after the end date of this project. We have not yet received the cells, as the staff at West Virginia University has not been able to prioritize their growth and authentication over science and protocols that are part of their response to COVID-19.

Specific Aim 3, Task 2, Subtask 1: Genetically modified cells will be created using resources available at the Masonic Cancer Center Genome Editing Shared Resource.

Initiation of these experiments was delayed because the lead technician in the Genome Engineering Shared Resource of the Masonic Cancer Center (UMN) had to take medical leave during 2019. Immediately upon his return, we were able to start the work to develop genetically modified cells as per our proposed milestones. We modified the human osteosarcoma cell line, HOS to express CD63-GFP using CRISPR-Cas9. The gene was targeted to the AAVS1 locus in the genome. Successful integration confirmed six clones expressed GFP from the AAVS1 locus by PCR analysis from both the 5' and 3' integration junctions. Clone HOS-17 was expanded and used for *in vivo* xenografts. However, the expression of a single copy of the fusion gene did not generate the same signal we observed using conventional transfection methods, which introduce multiple copies of the gene into the genome (see for example **Figure 22**). We were unable to visualize GFP-labeled exosomes in HOS-17 cells using fluorescence microscopy or flow cytometry.

We also used CRISPR/Cas9 to modify K12 and K7M2 cell lines to express Gaussia luciferase (Gluc) and chicken ovalbumin (OVA). Guide RNAs targeting the Rosa26 locus (5'-GAAGAUGGGCGGGAGUCUUC-3') were used to knock in a bicistronic cassette encoding both genes. We did not anticipate major obstacles, as we had previously used this same approach to knock in a bicistronic cassette encoding Gluc and GFP into MC-17 fibrosarcoma cell lines. The advantages of CRISPR/Cas9 knock-ins, versus other commonly used methods to generate stable G-Luc-positive and OVA-positive cells was the ability to control the number of copies

expressed (to be able to directly compare quantitative data from in vivo imaging and immune responses), as well as to limit genomic disruption to the targeted locus.

We confirmed successful knock-in of the cassette encoding Gluc and OVA into mouse K12 osteosarcoma cells using PCR (**Figure 36**). But to our surprise, these cells had about 40-fold lower luciferase activity than the previously engineered MC-17 cells encoding Gluc-GFP (**Figure 37**). It is still unclear if the co-expression of Gluc and OVA is responsible for this effect, if the Gluc gene was methylated in both clones, dampening its expression, or if another mechanism hindered Gluc activity in these cells. There are no epitope-specific antibodies that allow us to detect expression of OVA peptides on mouse H-2^d (the MHC class I haplotype for Balb/c), and an H-2^d, class I-restricted TCR against OVA has not been generated, precluding direct analysis of expression of OVA peptides on the surface of the modified K12 cells. While we could investigate intracellular OVA, for example using Western blots or mass spectrometry, this would not inform the potential for antigen presentation to cytotoxic T cells. And the inability to track the cells in vivo due to dim Gluc expression would also limit the interpretation of experiments.

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      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
I A V A E A K P T E N N E D F N I V A V A S N F A T T D L D A D R
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I V D I P E I P G F K D L E P M E Q F I A Q V D L C V D C T T G C
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Figure 36. Sequencing of in-frame OVA and G-Luc in K12 mouse osteosarcoma cell line clone 17. The green sequence in the 5' end of this sequencing reaction represents the full length chicken ovalbumin (OVA) gene integrated in frame as predicted, followed by a spacer sequence and the first 145 amino acids of the Gaussia luciferase protein (per design of the PCR amplification reaction).

Even more surprising to us was the fact that the K7M2 cells were extremely refractory to transfection for CRISPR/Cas9 modification. We chose Neon electroporation, which was used successfully for MC-17 and mouse K12 cells, as the transfection method. Transfections to integrate the constructs into K7M2 cells were attempted more than five times. After the first attempt, all of the cells died. The second attempt resulted in viable cells and testing of the bulk transfection supernatant showed Gaussia Luciferase activity. However, none of 44 subclones tested showed successful targeting of the Rosa26 locus. For the third attempt, we used an IDT HDR enhancer to improve expression, but once again, all the cells died. A fourth attempt was

done with and without the HDR enhancer. All of the HDR enhancer treated cells died while the non-treated had 50% viability. We subsequently attempted transfections with the HDR enhancer using a lower voltage for the electroporation with no better results. Alternative transfection protocols proved no more successful, even though the cells showed robust viability in culture prior to every transfection attempt. It is unclear if the problems we encountered lie in the cell lines themselves, or in the construct or the methodology, but these challenges might explain why, despite the widespread use of the two cell lines, there are no reports of K12-Luc (or K12-OVA)-expressing clones and only a single report of successful transfection of K7M2 cells with a Luc plasmid²⁷. We contacted the senior author from this manuscript at the end of 2019, and were referred to Dr. Brock Lindsey, the head of the Musculoskeletal lab at West Virginia University. Dr. Lindsey's head technician responded at the end of January 2020, indicating that they would agree to transfer the cells to our lab through an MTA. But the COVID-19 closures at both UWV and UMN delayed completion of the MTA until November 2020, which was two months after the end date for this project. We will continue to use these cells, however, for our ongoing project developing P-DIC immunotherapy.

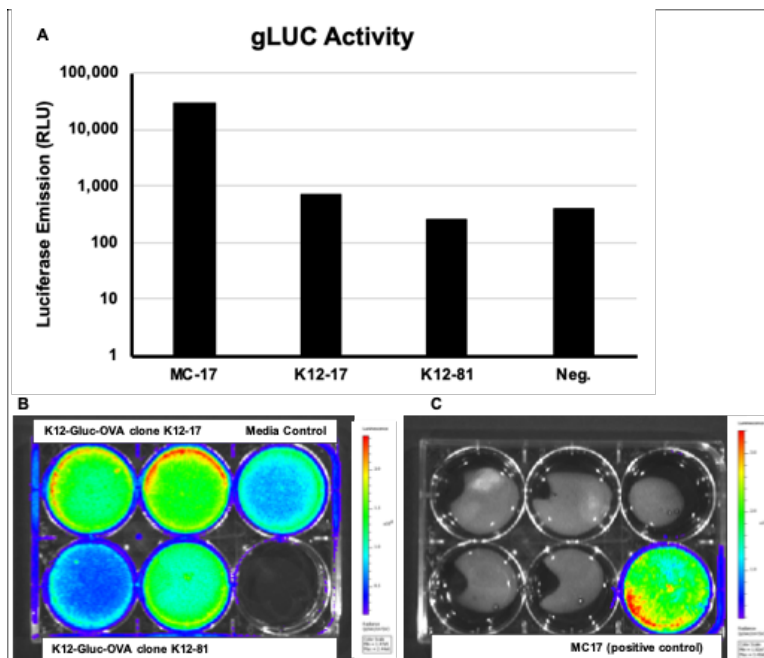


Figure 37 Luciferase activity in genetically-modified mouse K12 osteosarcoma cells. (A). Luciferase activity was measured *in vitro* in culture supernatants from MC-17-Gluc-GFP cells (positive control), K12-Gluc-OVA clones K12-17 and K12-81, and control media. One of the properties that makes Gaussia luciferase easy to work with is that it is secreted from the cells into the media or surrounding environment. The measured luciferase activity in MC17-Gluc-GFP cell supernatants was approximately 28,800 relative luciferase units (RLU). In contrast, the activity in K12 clone 17 (K12-17) supernatants was only approx. 700 RLU, which was 2X background, but still 40-fold lower than that observed in MC17. (B) Luciferase activity in K12-Gluc-OVA clones K12-17 and K12-81 measured *in vivo* (in cells), and in control media (no cells) using the IVIS Spectrum Imager. Note minimum and maximum radiance scales are 1.47×10^5 and 2.44×10^6 (photons/sec/cm²/sr). (C) Luciferase activity in MC17-Gluc-GFP (positive control) cells measured using the IVIS Spectrum Imager. Note minimum and maximum radiance scales are 1.82×10^7 and 3.40×10^8 (photons/sec/cm²/sr), consistent with the luminometer measurements.

Specific Aim 3, Task 3: Validation of PD-1/PD-L1 blockade *in vitro*.

We showed that P-DIC binds to cells expressing CD47 and PD-L1 and that it successfully competes with antibodies that bind CD47 and with antibodies that bind PD-L1.

Specific Aim 3, Task 3, Subtask 1: The PD-1 variants will be used to verify that they promote enhanced T-cell activation that is comparable to anti-PD-1 antibodies (mouse).

Some of the work shown in this section was done as a collaboration with Dr. Shruthi Naik at the Mayo Clinic.

We showed that P-DIC binds to human and mouse cells that express CD47 and PD-L1 (**Figures 38B, C, and D**). The binding to both species showed similar affinity, despite known differences in the sequence of the target proteins. This suggests that the recombinant P-DIC peptide retains important structural features that are evolutionarily conserved as part of the interactions between CD47 and its SIRP α and SIRP γ ligands and between PD-1 and its PD-L1 ligand. We also documented that P-DIC could displace antibodies against CD47 and against PD-L1 in human and mouse cells (**Figures 38C and D**). Our data show that P-DIC achieved >50% displacement of high affinity anti-PD-1 in 5TGM1 mouse myeloma cells and of high affinity anti-CD47 in mouse J774 macrophages at equimolar concentrations in competitive binding assays, despite

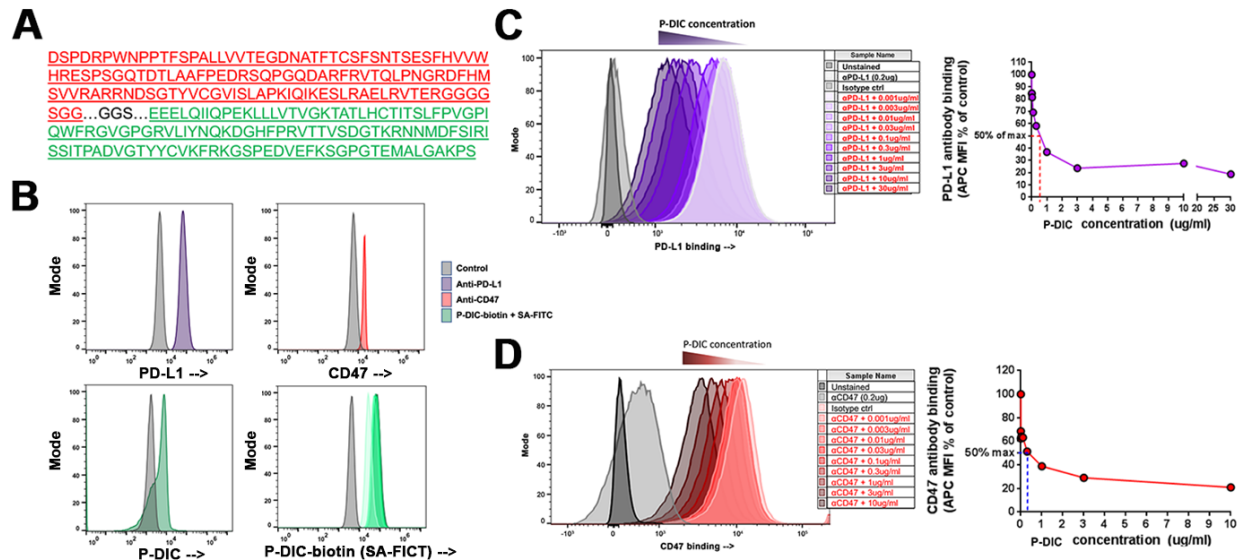


Figure 38. Structure and binding of P-DIC to PD-L1⁺ and CD47⁺ cells. (A) Secondary structure (sequence) of P-DIC. N-terminal red sequence encodes the soluble high affinity PD-1 consensus sequence; black sequence encodes the spacer; C-terminal green sequence encodes the high affinity SIRP γ gamma variant-3 domain. (B) Binding of P-DIC to human PD-L1⁺/CD47⁺ A549 cells. Top left: expression of PD-L1 (saturating anti-PD-L1 antibody). Top right: expression of CD47 (saturating anti-CD47 antibody). Bottom left: binding of biotinylated P-DIC. Bottom right: saturation curve of biotinylated P-DIC with increasing concentrations of streptavidin (SA)-FITC. (C) Blockade of anti-PD-L1 binding by P-DIC. PD-L1⁺ 5TGM1 mouse myeloma cells were incubated with 0.2 μ g/mL anti-PD-L1 antibody and increasing concentrations of P-DIC. The plot on the right shows the concentration dependent displacement of labeled anti-PD-L1. (D) Blockade of anti-CD47 binding by P-DIC. CD47⁺ J774 mouse macrophages were incubated with 0.2 μ g/mL anti-CD47 antibody and increasing concentrations of P-DIC. The plot on the right shows the concentration dependent displacement of labeled anti-CD47.

binding both receptors simultaneously. This suggests CD47- and PD-1 blockade with P-DIC would be at least as effective as that achieved with antibodies against these molecules. We generated preliminary data suggesting that P-DIC enhances phagocytosis of tumor cells by normal macrophages, although completion of this task, specifically to verify enhanced phagocytosis enhanced T-cell activation by P-DIC, were delayed by the COVID-19 pandemic and our inability to conduct any experiments between March and July of 2020, and the tight restrictions and shortages of supplies we experienced between July and August of 2020 when the project ended.

Specific Aim 3, Task 4: Optimization of cell dose and tumor growth kinetics for K12 and K7M2 osteosarcoma cell lines.

The initial work on this task was delayed because of the problems generating genetically modified cells. Given these concerns and the timelines for the project Dr. Naik, at the Mayo Clinic, initiated experiments to establish growth of K7M2 cells as heterotopic tumors injected under the skin. The development of these tumors in mice using previously published cell numbers was exceedingly long (more than 90 days).

Specific Aim 3, Task 4, Subtask 1: We will optimize the orthotopic syngeneic graft model using cells that express luciferase, CD63-GFP, +/- OVA in groups of 3 mice.

Many studies have been done using subcutaneous injection of mouse osteosarcoma cells K12 and K7M2, but less work has been done using orthotopic implantation. We secured collaboration from Dr. Branden Moriarity and Mr. Branden Smeester who have extensive experience with orthotopic implants of osteosarcoma in mice. In the past, investigators have generally used chemical anesthesia for these procedures, but Mr. Smeester, working initially with Dr. Al Beitz and subsequently with Dr. Moriarity perfected protocols for orthotopic implantation of bone tumor cells into the calcaneus bone, which could be done under general gas (isoflurane) anesthesia, reducing the induction and recovery time, as well as the potential for anesthetic side effects and deaths. The procedure was subsequently adapted to intratibial injections, which our group had only previously performed using general chemical anesthesia.

We completed training sessions with Mr. Smeester and Dr. Nate Koewler (Research Animal Resources area veterinarian) and confirmed that the intratibial implantation was feasible under general gas anesthesia, with 100% survival and no evidence of anesthetic side effects. Intratibial implantation was also more efficient to generate tumors within the medullary cavity, as compared to implantation into the calcaneus, which showed greater potential for growth in the periosteum and which may alter the patterns of spontaneous metastasis.

Delays in obtaining labeled cells and the limited access to the lab caused by the COVID-19 pandemic precluded completion of the experiments to establish the orthotopic model.

Specific Aim 3, Task 5: Stabilized sPD-1 dose finding in mice.

We have data to show that CD47 blockade is efficacious to promote osteosarcoma phagocytosis *in vivo*. The dose-finding experiments for P-DIC, however, were delayed due to the COVID-19 response and have resumed to be included as part of the work that will be done for our recently funded Impact Award.

Specific Aim 3, Task 5, Subtask 1: We will define any local and systemic toxicity attributable to the stabilized sPD-1 variant and establish a safe dose using normal mice with a standard design (evaluating 4 doses in 5 animals per dose).

As part of a previously established collaboration with Dr. Irv Weismann's group at Stanford University, we had examined the potential to target CD47 blockade as treatment for osteosarcoma. These data, which remain, were important to support the notion of dual immune checkpoint blockade. For those experiments, we used a xenograft model to evaluate the therapeutic potential of this combination. OSCA-40 osteosarcoma cells were injected subcutaneously into immunodeficient (NSG) mice. Treatment with anti-CD47 blockade

was initiated two weeks after engraftment and continued for six weeks. There was no evidence of toxicity associated with CD47 blockade in this model. Furthermore, not only did the mice receiving CD47 blockade therapy outlive those that received sham therapy, but they also showed decreased lung metastasis, as evaluated by *in vivo* bioluminescence imaging (**BLI**; **Figure 39**).

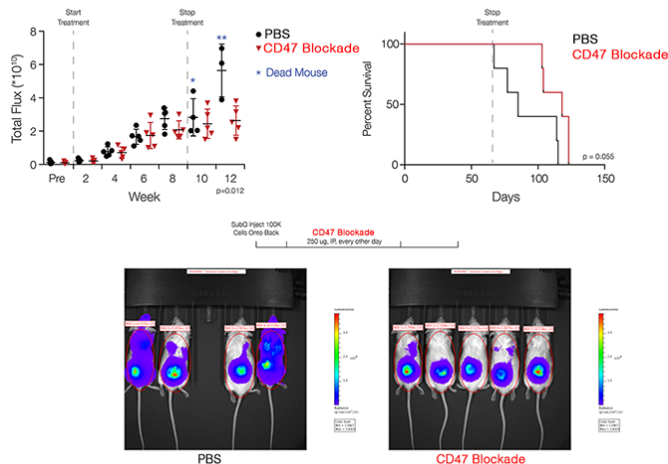


Figure 39. CD47 blockade in osteosarcoma. NSG mice were inoculated with 100,000 highly metastatic OSCA-40 cells. One week later, mice received CD47 blockade therapy (250 µg IP every other day) for 8 weeks. A control group of mice received PBS in the same volume and on the same schedule. The top left panel shows survival for each group of mice (based on tumor endpoints), with mice receiving CD47 blockade shown in red and mice receiving PBS shown in black. One mouse died before it could be humanely sacrificed (PBS group, week 10). The top right panel shows the Kaplan Meier survival plot, showing a survival advantage for the group treated with CD47 blockade. BLI (bottom panels) shows reduced or delayed pulmonary metastasis in the group receiving CD47 blockade.

Dr. Naik's group was able to document an immune response could be generated against K7M2 cells in the subcutaneous model using VSV (**Figure 40**), but as was the case for other aspects of this specific aim, the response to the COVID-19 pandemic delayed our joint ability to complete dose finding studies for P-DIC efficacy in mice. Those experiments have been resumed as part of our recently funded DOD Impact Award and will be reported as part of our annual report for that project.

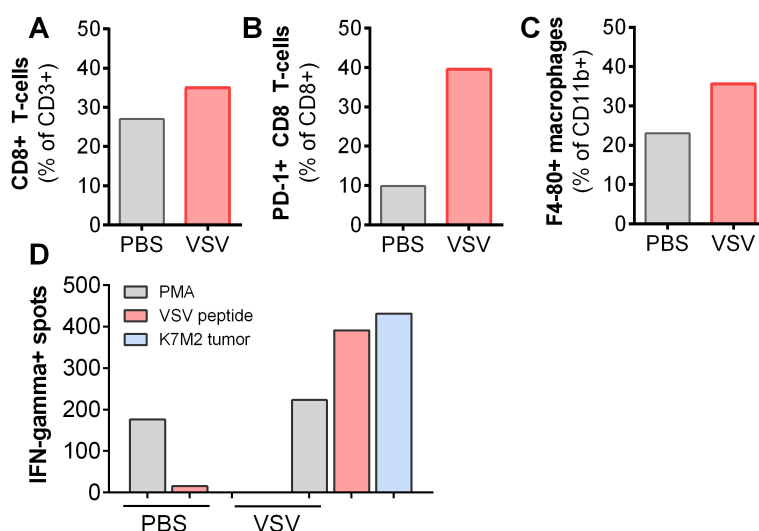


Figure 40. Immunostimulatory activity of VSV in the K7M2 murine osteosarcoma model. Mice bearing subcutaneous K7M2 tumors were treated with a single dose of 1×10^8 VSV. Mice were sacrificed 7 days post treatment and peripheral blood and spleen were collected. Peripheral blood mononuclear cells were isolated and analyzed by flow cytometry showing (A) a small increase in CD8 T cells (as a proportion of all T cells), (B) an increase in activated (PD-1⁺) CD8 T-cells, and (C) an increase in F4-80⁺ macrophages (as a proportion of CD11b⁺ myeloid cells) was noted in the experiment. (D) Splenocytes were isolated and tested in an IFN γ ELISPOT assay showing an increase in VSV-specific and tumor-specific cytokine production in VSV-treated mice compared to PBS (no stimulation control subtracted from all values). PMA (phorbol myristate acetate) was used as a positive control for stimulation.

Specific Aim 3, Task 6: *In vitro* immunological assays.

These experiments were not completed due to the delays we encountered generating OVA-expressing cells that were further compounded by the COVID-19 pandemic and response, as mentioned above.

Specific Aim 3, Task 6, Subtask 1: We will separate tumor exosomes (CD81/GFP+) from host exosomes (CD81/GFP-) in serum by flow sorting.

These experiments were not completed due to the poor signal encountered in the HOS-17 (CD63-GFP) cells and the inability to resume work to create additional cell lines due to the COVID-19 pandemic and response, as mentioned above.

Specific Aim 3, Task 6, Subtask 2: We will evaluate CD28 expression in T-cells isolated from tumors and lymph nodes, as well as T-cell activation after re-stimulation *in vitro* by measuring IL-2 and interferon- γ production using ELISpots (or flow cytometry).

The experiments to assess CD28 expression were deferred based on the data from Specific Aim 1 and Specific Aim 2. Preliminary data for the immune activation experiments are shown in Figure 40. However, these latter experiments were not carried to completion due to the delays we encountered generating genetically engineered cells for *in vivo* stimulation, which was further compounded by the COVID-19 pandemic and response, as mentioned above.

Specific Aim 3, Task 7: *In vivo* immunological assays.

These experiments were not completed due to the delays we encountered generating OVA-expressing cells that were further compounded by the COVID-19 pandemic and response, as mentioned above.

Specific Aim 3, Task 7, Subtask 1: We will define antigen specific responses in syngeneic mice using OVA-expressing tumors and adoptively transferred DO11.10 T cells. Specifically, we will quantify T cells in tumors by flow cytometry using the KJ1-26 antibody. Groups of 10 mice will be used for each experiment.

See above. We were unable to initiate experiments for this task.

Specific Aim 3, Task 7, Subtask 2: We will estimate migration of T-cells to tumors by IHC, staining for egress markers in lymphocytes and blood vessels. Adoptively transferred T-cells will be distinguished from host T-cells using congenic markers.

See above. We were unable to initiate experiments for this task.

Specific Aim 3, Task 7, Subtask 3: We will evaluate proliferation of antigen specific T-cells in the draining and non-draining (contralateral) lymph nodes using dye dilution assays.

See above. We were unable to initiate experiments for this task.

What opportunities for training and professional development has the project provided?

1. Dr. Anne Sarver worked on this project (RNA and microRNA analysis) as part of her post-doctoral fellowship and her Masters in Clinical Research, supported through the Pediatric Cancer Epidemiology Training Grant of the University of Minnesota (T32 CA099936, Dr. Logan Spector, PI). Dr. Spector and Dr. Modiano were co-mentors for Dr. Sarver's fellowship. Dr. Sarver defended her Master's thesis and is now a Principal Scientist in the Clinical Research Division at Abbott/St. Jude.

2. Dr. Kelly Makielski worked on this project (RNA and exosome isolation, immune response) as part of her post-doctoral fellowship, supported through the Cancer Biology Training Grant of the University of Minnesota (T32 CA009138, Dr. Carol Lange, PI). Dr. Modiano is Dr. Makielski's post-doc mentor. Dr. Makielski has completed her NIH-supported post-doctoral fellowship and has applied for a mentored, career development grant (K01), based partly on work done for this project, to support her transition to independence.

3. Dr. Jong Hyuk Kim is a former post-doc/research associate who was also key personnel working on this project (bioinformatics and pathology). Dr. Kim was promoted to Assistant Professor in the Department of Veterinary Clinical Sciences of the University of Minnesota College of Veterinary Medicine in 2018.

How were the results disseminated to communities of interest?

The work done for this project was disseminated to the scientific community through publication of data as peer reviewed manuscripts in scientific journals (one paper published, one in revision, and three in preparation) and by abstracts and poster presentations at local and national meetings. Two articles about our research, including our current understanding of cancer risk and the contributions of studies in dogs to that understanding, were published in electronic media aimed at enhancing scientific literacy among the general public and government officials.

Peer reviewed publications:

1. Mills, L. J. et al. Comparative analysis of genome-wide DNA methylation identifies patterns that associate with conserved transcriptional programs in osteosarcoma. *Bone*, 115716, doi:10.1016/j.bone.2020.115716 (2020). *Included in the appendix.*
2. Xiao, X. et al. Tumor secreted extracellular vesicles regulate T-cell costimulation and can be manipulated to induce tumor specific T-cell responses. *Gastroenterology*, in revision. *Included in the appendix.*

Manuscripts in preparation:

1. Donnelly, A. et al. Development of an exosomal biomarker signature to detect minimal residual disease in dogs with osteosarcoma using a novel xenograft platform and machine learning.
2. Sarver, A. E. et al. Transcriptional networks controlling osteosarcoma progression.
3. Mills, L. J. et al. Structural genomic rationale for the increased vulnerability to osteosarcoma in dogs.

4. Modiano, J. F. and Sarver, A. L. Evolutionary constraints on longevity and body size regulate cancer risk and provide a species-specific resolution to Peto's Paradox.

Meeting abstracts and poster presentations:

1. Modiano, J. F. et al. (2019). Cancer as a Consequence of Breaking Through Evolutionary Constraints on Longevity. Proceedings of the American Association for Cancer Research.
2. Troske, D. et al. (2019.) Down-regulation of CD28 Through Exosomes Secreted by Osteosarcoma. Masonic Cancer Center Genetic Mechanisms of Cancer Program Retreat, Minnesota Landscape Arboretum, Chaska, MN, June 12, 2019
3. Schulte, A. J. et al. (2020). Impact of tumor-derived exosomes on CD28 expression in T cells. Proceedings of the AACR Special Conference, Advances in Liquid Biopsies. Clin Cancer Res, Volume: 26, Issue: 11 Supplement: S Pages: 51-52 Meeting Abstract: A33

Articles in lay educational online platforms:

1. Modiano, J. F. Understanding Cancer Development in Humans and Their Companion Animals. *Scientia Global*, October 22, 2019 <https://doi.org/10.33548/SCIENTIA425>
2. **Modiano JF** (2019). Cancer in Dogs and Humans: How is it alike and how is it different? *Open Access Government*, April 2020

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

We had previously described convergent molecular (transcriptional) programs that define the biology of osteosarcoma, and in particular, the heterogeneity of immune responses and their role in outcome for patients with this cancer. Data from this project have expanded our understanding of the mechanisms that drive this convergence. Specifically, we have been able to clarify important differences in risk and causation of bone cancer in humans and dogs, introducing a cautionary note about the use of dogs as "models" to study the etiology of these tumors. On the other hand, we have solidified the similarities once tumors form, creating homologous structures with comparable organization and molecular programs, which allow us to explore how the tumors achieve this peculiar organization, and in particular, how osteosarcomas establish an immunosuppressive barrier that mediates resistance to innate (pre-existing in the patient) and induced (therapeutic) immune responses directed against these tumors.

In the short term, the information arising from this project will help us inform potential patient outcomes (prognosis). In the medium term, it has yielded a new and unique biological molecule that may find a role in the treatment of osteosarcoma. In the long term, it will help us develop more effective, personalized therapies for children, adolescents, and young adults with bone cancer. An additional (bonus) benefit of this research will be improvements in the way we

diagnose, treat, and ultimately prevent bone cancer in companion and service dogs, which can be significant in improving the health, welfare, longevity, and performance of military working dogs.

What was the impact on other disciplines?

The GCESS method of defining transcriptional programs was a first-of-its kind analysis. This method extends to other tumors, as well as to other complex medical conditions that include heterogeneous cell populations. Expanding this to analyze genome wide next generation microRNA sequencing and methylome datasets was, again, innovative, and will be able to be extended to other tumors and other diseases. The mechanistic links between transcriptional programs (mRNA and microRNAs) and between gene expression and chromatin accessibility were predictable; but tools to examine these concurrently in dogs with spontaneous cancer that can serve as models for human disease were developed as part of this project and will be and widely applicable to many areas of biology. All of this, together, will help us analyze how exosomes and their cargo affect tumor biology locally and systemically and enable predictions of response to immune modulation in patients with multiple cancers and other non-malignant diseases.

What was the impact on technology transfer?

The exosome biomarker discovery platform and an exosomal mRNA signature for detection of minimal residual disease in dogs with osteosarcoma were subjects of two patents filed through the University of Minnesota where the inventors were part of the investigator team for this project. These technologies are part of the intellectual property that enabled the launch of Half Moon Bay Biotechnology, LLC., a start-up company from the University of Minnesota Discovery Launchpad that is focused on development of actionable laboratory tests for companion animals with cancer.

The P-DIC molecule was licensed by Yale University to Ligand, Inc., which in turn sublicensed it to Zymeworks, Inc. We have initiated discussions with the management at Zymeworks to ensure that the product will be available for future clinical development.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change:

The COVID-19 pandemic had considerable, negative effects on our capacity to conduct laboratory work during much of 2020. The University of Minnesota, along with much of the State of Minnesota, implemented a shelter-in-place (aka, “lockdown”) starting on March 16, 2020. Every lab at the University of Minnesota that was not considered “essential” based on

doing COVID-19-related work was ordered to go into hibernation and all employees were required to work remotely. As part of our hibernation plan, all cells were frozen down, all genome engineering and sequencing experiments were suspended, and no new animal experiments could be started. A member of the lab was allowed to go in for approximately 1 hour twice each week to fill our liquid nitrogen tanks and to make sure critical equipment, like refrigerators and freezers, was in working order.

Work on cell culture models in our lab only resumed in July of 2020. We were able to have some of our animal model experiments done by our collaborators at the Mayo clinic in Rochester, MN (at no cost to this project). As of the end of this project (August 31, 2020), the lab continued to operate under a “Sunrise plan” that allowed only approx. 25% of the scheduled activities to be performed. Staff mostly worked remotely, whenever possible, or when in lab, they mostly kept non-overlapping schedules to minimize contact.

Some segments of the University were able to resume partial work on projects as time permitted. For example, the University of Minnesota Genome Center (UMGC) resumed deferred sequencing work late in April of 2020. On the other hand, the Genome Engineering Shared Resource core of the Masonic Cancer Center did not resume work on deferred projects until late July of 2020 and the Bionet Histology Laboratory did not resume work until August of 2020.

This had a major impact on our progress. Staff continued to be paid. Remote work revolved around data analysis and hypothesis-generation. Some of the data analyses in this report are still ongoing (even though the project is completed) because data were delayed. Even so, the investigators believe the program officers and reviewers will agree we made considerable progress towards the stated goals of the project.

Modifications to project aims, tasks, and milestones.

1. RNA sequencing of lymph nodes: We did not sequence mRNAs and microRNAs for lymph nodes of dogs with osteosarcoma. Frozen lymph nodes were collected as part of an independent clinical study in dogs supported through independent funding (from the State of Minnesota and Vyriad, Inc.) that was ongoing concurrently with this project. The main objective for the lymph node samples was to examine lymphocyte clonality in dogs after treatment with an oncolytic virus. We completed sequencing for tumor tissues and for some of the cell lines but given the budget constraints of the project and the challenges of fitting work in during the COVID-19 pandemic, we were unable to complete RNA sequencing of bulk lymph node samples. Sequencing efforts for the lymph node samples were limited to DNA sequencing to determine if there were changes in the abundance or distribution of lymphocyte clones.

2. Number of new samples subjected to RNA sequencing: We proposed to sequence 50 new tumor samples from dogs with osteosarcoma. We identified 62 samples that met the initial criteria for inclusion in the project. However, as described in the narrative for section 3, only 43 samples met the QC metrics for mRNA sequencing and only 28 samples met the QC metrics for small RNA sequencing. Delivery of sequencing data was also impacted by the COVID-19 pandemic.

3. Addition of genome-wide methylation sequencing: Because our data suggested there was not a strong regulatory link between expression of microRNAs and the immune landscape in osteosarcoma, we explored whether the transcriptional programs that are evident in this disease might be regulated by chromatin accessibility. We used a commercial kit to process human samples, and working with Roche/Nimblegen, we developed a new bait capture kit for canine genome-wide methylome analysis (with approx. 5 Mb resolution) that would be available for Roche to commercialize. The data are published, but at this time, Roche has decided not to include this as a kit in their catalog.

4. Statistical correlations of exosome microRNAs and immune transcript abundance in tumors and lymph nodes: This task was deferred due to technical obstacles generating exosomes from preserved, frozen tissues and the likely futility of performing the analyses in cultured cells, as described in the narrative for section 3. We identified a kit that might achieve reliable exosome enrichment from frozen tissues (the Capture EM kit) late in the summer of 2020. However, because of limitations on lab work during the pandemic, we were unable to test whether this method would provide suitable preparations to complete this subtask. As noted above, we deprioritized analysis from *in vitro* cultures and we did not perform RNA sequencing from lymph nodes.

5. Building a tissue microarray, completing quality control metrics, and using the resource to establish the presence of immune cells in tumors: The TMA was built, but the COVID-19 pandemic delayed its completion until after the project had ended. QC has been performed and this resource will be available for future investigations. IHC to interrogate osteosarcoma samples for the presence of immune cells was done as part of a previous, related study and the data are included in the narrative for section 3.

6. Generate Dicer knockout and microRNA knockdown osteosarcoma cell lines. Cells with knockdowns of CD28-targeting microRNAs were generated for colorectal cancer models. But given our data showing no correlation between CD28-targeting microRNAs and immune regulation in osteosarcoma, we did not generate Dicer knockout or microRNA knockdown osteosarcoma cell lines for this project.

7. Testing mouse cells for CD28 downregulation: Given our data showing that CD28 was stable in human and canine T cells cultured in the presence of exosomes, we decided not sacrifice mice for these experiments, as we considered them futile. This is consistent with our efforts to eliminate unnecessary use of animals for research.

8. Experiments using amiloride to inhibit exosome production: the general absence of effects from exosome preparations on T cells, as well as the observation that in the one instance where an effect was observed it was only seen with species-relevant exosomes and not with mixed-species exosomes made the amiloride experiments predictable and unnecessary.

9. Dye dilution experiments: We did not observe differences in viability or cell counts in experiments where T cells were co-cultured with and without exosomes, so we did not perform

dye dilution experiments, as we believed such experiments were unnecessary in light of the negative data.

10. T-cell migration experiments: As noted in the narrative of section 3, these experiments were similarly considered likely to yield little or no useful information based on the complement of data from the project. We did observe, however, a modest effect of exosomes to accelerate migration (and proliferation) of non-immune stromal cells, as well as alterations in the gene expression patterns, suggesting that one of the ways in which exosomes alter the immune landscape is by reducing production of inflammatory and chemotactic cytokines by stromal cells.

11. Production of *in vivo* grade high affinity stabilized sPD-1 variant: we generated a bispecific peptide to block not only the interactions between PD-1 and PD-L1, but also the interactions of CD47 with natural ligand, SIRP α .

12. Definition of a dose for sPD-1 in mice: Completion of these experiments was hindered by the COVID-19 lockdowns and our inability to initiate animal experiments for much of 2020. We have data to support an effect for P-DIC and the experiments to establish safe and effective doses are ongoing as part of a funded Impact Award.

13. *In vivo* experiments for aim 3 and *in vitro* validation of immune reconstitution in tumors: We were unable to complete these experiments due to two separate but interrelated obstacles. One was our inability to generate the necessary genetically-engineered cells for the orthotopic models. This is the first time that we have failed in generating genetically-modified cells using CRISPR-Cas9. In two instances (human HOS cells modified to express CD63-GFP and mouse K12 cells modified to express OVA-Gluc), we documented integration of the cassettes into the right locus, but the genes were not expressed at levels that were suitable for our experimental needs. In one instance (K7M2 mouse cells), we simply could not get the cassette into the target locus or the cells to survive transfection. We remain puzzled by this result but have resorted to obtaining K7M2 cells that stably express Luciferase from a lab at West Virginia University. Regrettably, the COVID-19 response also delayed our ability to obtain these cells in a timely manner for the project.

Actual or anticipated problems or delays and actions or plans to resolve them

There were unanticipated changes in non-key personnel.

- Dr. Anne Sarver completed her Masters in Clinical Research and took a job at Abbott/St Jude.
- Ms. Lisa Basso, a technician in Dr. Subramanian's lab that had been assigned significant effort on the project, left the lab for another job before she could contribute to our efforts. To compensate, Dr. Subramanian increased his effort from the proposed 8% (0.96 person cal months) to approx. 11% (1.32 person cal months).
- Mr. Derek Troske, a technician in Dr. Modiano's lab, was responsible for significant effort on this project. Unfortunately, Mr. Troske left the lab at the end of the year due to issues with

under-performance. His effort was re-distributed among three senior technicians in the laboratory that had between three and eight years of experience and a long history of excellent reliability.

- Mr. Brian Ruis, the technical director of the Genome Editing Shared Resource had a medical problem and had to take temporary leave. Mr. Ruis returned full time by the beginning of year-2 of the project, but this created delays in the generation of proposed genome-edited cell lines. Additional problems with the generation of genetically engineered cell lines are described above.

The University of Minnesota Genomics Center replaced their main “workhorse” HiSeq-2500 sequencers with a new Novaseq. While this was generally great for our science, there were delays during the replacement, and the model for the Novaseq required more samples to fill lanes, so the turnaround time for sequencing was considerably slower than what we were used to. This problem was compounded by the advent of the COVID-19 pandemic, which required the UMGC to refocus virtually all their effort from March until almost June of 2020 on projects for COVID-19 testing.

Aims and tasks labeled as “ongoing” are continuing funded through related projects to extend these investigations or through the investigators’ discretionary sources. Support from DOD Award will be included when reporting on these efforts.

Changes that had a significant impact on expenditures

Changes in expenditures were largely maintained within categories. Details will be provided in the final financial status report. For example, the change in effort for Dr. Subramanian moved funds from the Professional and Academic line (senior research specialists and non-tenure track faculty) to the tenured/tenure track faculty line. Cost of living and merit raises for staff required minimal of reductions of effort for senior investigators to allow for maintaining the full effort for research staff. Funds that were allocated to mouse purchases or to per diems were reallocated to other medical services (sequencing) or to purchase supplies (culture reagents, antibodies, etc.) as needed.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

None

Significant changes in use or care of vertebrate animals

As described above, some of the planned experiments using mice were not done. This did not impact any animals, since the mice were not purchased, and therefore they were not used for experiments. This partly followed scientific rationale and appropriate efforts to reduce the unnecessary use of animals in research. But partly, it was due to the impact of the COVID-19 pandemic and the response to the pandemic, which precluded on-site experimentation for a significant portion of the calendar year 2020, and virtually 50% of the duration of the second year of the project.

Significant changes in use of biohazards and/or select agents

None

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Peer reviewed publications:

1. Mills, L. J. et al. Comparative analysis of genome-wide DNA methylation identifies patterns that associate with conserved transcriptional programs in osteosarcoma. *Bone*, 115716, doi:10.1016/j.bone.2020.115716 (2020). (This manuscript acknowledges federal support) *Included in the appendix.*

2. Xiao, X. et al. Tumor secreted extracellular vesicles regulate T-cell costimulation and can be manipulated to induce tumor specific T-cell responses. *Gastroenterology*, in revision. (This manuscript acknowledges federal support) *Included in the appendix.*

Manuscripts in preparation:

1. Donnelly, A. et al. Development of an exosomal biomarker signature to detect minimal residual disease in dogs with osteosarcoma using a novel xenograft platform and machine learning. (This manuscript will acknowledge federal support).

2. Sarver, A. E. et al. Transcriptional networks controlling osteosarcoma progression. (This manuscript will acknowledge federal support).

3. Mills, L. J. et al. Structural genomic rationale for the increased vulnerability to osteosarcoma in dogs. (This manuscript will acknowledge federal support).

4. Modiano, J. F. and Sarver, A. L. Evolutionary constraints on longevity and body size regulate cancer risk and provide a species-specific resolution to Peto's Paradox. (This manuscript will acknowledge federal support).

Books or other non-periodical, one-time publications. .

None

Other publications, conference papers and presentations

Meeting abstracts and poster presentations:

*1. Modiano, J. F. et al. (2019). Cancer as a Consequence of Breaking Through Evolutionary Constraints on Longevity. *Proceedings of the American Association for Cancer Research*. Presented as a poster at the annual meeting in 2019. This poster acknowledged federal support and a manuscript is in preparation (see above).

2. Troske, D. et al. (2019.) Down-regulation of CD28 Through Exosomes Secreted by Osteosarcoma. Masonic Cancer Center Genetic Mechanisms of Cancer Program Retreat, Minnesota Landscape Arboretum, Chaska, MN, June 12, 2019. Presented as a poster at the annual Masonic Cancer Center, University of Minnesota Genetic Mechanisms of Cancer Program retreat in 2019.

3. Schulte, A. J. et al. (2020). Impact of tumor-derived exosomes on CD28 expression in T cells. Proceedings of the AACR Special Conference, Advances in Liquid Biopsies. Clin Cancer Res, Volume: 26, Issue: 11 Supplement: S Pages: 51-52 Meeting Abstract: A33. Presented as a poster at the first AACR meeting on liquid biopsies in 2020. This poster acknowledged federal support.

- **Website(s) or other Internet site(s)**

Articles in lay educational online platforms:

Modiano, J. F. Understanding Cancer Development in Humans and Their Companion Animals. *Scientia Global*, October 22, 2019 <https://doi.org/10.33548/SCIENTIA425>

Modiano JF (2019). Cancer in Dogs and Humans: How is it alike and how is it different? *Open Access Government*, April 2020. <https://www.openaccessgovernment.org/cancer-in-dogs-and-humans/83915/>

- **Technologies or techniques**

We developed a method to interrogate the canine methylome in collaboration with scientists at Roche/Nimblegen. Roche retains all rights to commercialize and distribute this project. At the present time, it is not actively listed in their catalog.

- **Inventions, patent applications, and/or licenses**

The Regents of the University of Minnesota have two filed patents related to work done for this project, but the provisional patents were filed prior to the start of the project (US Patent Application 15/783,776 filed on October 13, 2017) or the data were initially collected with support from another agency (US Patent Application 16/600,486 filed on October 12, 2019).

- **Other Products**

Dataset:

Comparative analysis of genome-wide DNA methylation identifies patterns that associate with conserved transcriptional programs in osteosarcoma

Organism: Homo sapiens; Canis lupus familiaris

Type: Methylation profiling by high throughput sequencing

Platforms: GPL16540 GPL11154 68 Samples

Download data: BED, TXT, XLSX

SeriesAccession: GSE149679ID: 200149679

Dataset:

Canine mRNA and microRNA sequencing

Will be submitted to GEO for release at the time of publication

Cell lines:

All cell lines generated through or used in this project that are not owned or licensed to other agencies will be made available either through Kerafast, Inc. or through institutional MTAs.

Tissue microarray:

A canine tissue microarray was generated. We estimate the array will yield between 40 and 60 slides. Slides will be made available to investigators for projects that are likely to advance our understanding of osteosarcoma through collaboration (preferred) or through institutional MTAs. However, the TMA blocks will not be sent outside of the University of Minnesota.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

*Name: Jaime Modiano
No change*

*Name: Subbaya Subramanian
No change*

*Name: Michael Farrar
No change*

*Name: Daniel Vallera
No change*

*Name: Brenda Weigel
No change*

*Name: Jong Hyuk Kim
No change*

*Name: Lauren Mills
No change*

*Name: Aaron Sarver
No change*

*Name: Anne Sarver
No change (Dr. Sarver completed her Master's project and left the University in 2019)*

*Name: Kelly Makielski
No change*

*Name: Aaron Ring
No change*

Other collaborators (no paid effort and no subcontract):

The following scientists have worked with us on experiments that were specifically proposed as part of the project, or on experiments that were related to the project and are included here for context, even though they were supported by other agencies.

*Name: Shruthi Naik
Institution: Mayo Clinic*

*Name: Brad Bryan
Institution: Texas Tech University*

*Name: Stephen Willingham
Institution: Stanford University*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Completed Grants (2018 - 2020)

Agency: National Institutes of Health/National Cancer Institute

Grant number: F30 CA195973

Principal Investigator: K. L. Anderson

Role: Mentor

Title of project: “Sensitization to CD47 blockade to enhance anti-tumor immunity”

Dates: 06/15/15 - 06/14/19

Agency: National Institutes of Health/National Cancer Institute

Grant number: R21 CA208529

Role: Principal Investigator

Title of project: “(PQ3) Modulation of Osteosarcoma Biology by Inflammation and Immunity Defined through a Comparative Approach”

Dates: 09/01/16-08/31/19 (NCE)

Agency: Minnesota Partnership for Biotechnology and Medical Genomics

Grant number: MNP #15.25

Role: co-Principal Investigator

Title of project: “Characterization of Anti-Tumor Immune Responses Following Oncolytic Virotherapy in Spontaneous Cancer”

Dates: 03/28/16 - 12/31/19

Agency: National Institutes of Health/Office of the Director
Grant number: K01 OD017242
Role: Mentor
Principal Investigator: Antonella Borgatti
Title of project: “Preclinical evaluation and clinical translation of novel bispecific toxins”
Dates: 05/01/14 - 04/30/19 (no cost extension to 05/31/2020)

Agency: National Institutes of Health/National Cancer Institute
Grant number: T32 CA009138 (individual fellowship within an institutional training grant)
Role: Mentor
Principal Investigator: Carol Lange
Fellow: Kelly Makielski
Title of project: “The VIGOR Clinical Trial: Induction of Anti-Tumor Immunity and Effects on Survival of Neoadjuvant Oncolytic Virotherapy in Dogs with Osteosarcoma”
Dates: 07/19/17 - 07/18/20

Agency: Vyriad, Inc.
Grant number: none assigned
Role: Principal Investigator
Title of project: “Translational Approach for Development of a Novel Oncolytic Immunotherapy for Osteosarcoma”
Dates: 05/01/16 - 12/31/19

Agency: Boston Scientific
Grant number: none assigned
Role: Principal Investigator
Title of project: “Alternating Electric Field Therapy to Disrupt Cancer for Immunotherapy”
Dates: 01/01/18 - 12/31/19

Agency: Boston Scientific
Grant number: none assigned
Role: co-Investigator (Biological Sciences Project leader)
Title of project: “COBALT: Biosensors for Early Disease Detection”
Dates: 03/01/15 – 06/30/20

Agency: AKC Canine Health Foundation
Grant number: 02234-MOU
Role: Principal Investigator
Title of project: “A Novel Approach for Prevention of Canine Hemangiosarcoma,”
Dates: 03/01/16 - 02/29/20

Agency: Morris Animal Foundation
Grant number: D18CA-050
Role: Principal Investigator
Title of project: “Mechanism of Action of a Novel, Safe and Effective Ligand Targeted Therapy to Define the Repertoire of Tumor Targets”

Dates: 12/01/17 – 11/30/20

New grants (2018 – 2020)

Agency: AKC Canine Health Foundation

Grant number: CHF Grant 02655-E

Principal Investigator: Caroline Wood

Role: Mentor

Title of project: “2019 Clinician-Scientist Fellowship - University of Minnesota”

Dates: 01/01/19 - 12/31/20 (NCE)

Total Direct Costs:

Effort: 0.12-person-calendar months

Goals: *This is a fellowship to support Dr. Wood’s work to define epigenetic signatures for canine lymphocyte subsets*

Overlap: None

Agency: The V Foundation

Grant number: T2018-018

Role: Principal Investigator

Title of project: “Novel Immunotherapy for Sarcomas using Combination Oncolytic VSV and IL-18 Superkine”

Dates: 03/01/19 - 03/28/22

Total Direct Costs:

Effort: 0.24-person-calendar months

Goals: *The goal is to define safety and efficacy of immunotherapy that combines an oncolytic virus to initiate an immune response with an IL-18 analog to expand and sustain the response*

Overlap: None

Agency: National Institutes of Health/Office of the Director

Grant number: K01 OD027058

Principal Investigator: S. Friedenberg

Role on Project: co-Mentor

Title of project: “Immunological Basis of Autoimmune Addison's Disease in a Novel Canine Model System”

Direct Costs:

Period of Award: 01/01/19 - 12/31/23

Overlap: None

Agency: University of Minnesota College of Veterinary Medicine, Comp Med Signature Program

Grant number: N/A

Principal Investigator: B. Walcheck

Role on Project: co-Investigator

Title of project: “Genetically Modified NK Cells for Canine Cancer Immunotherapy”

Direct Costs:

Period of Award: 01/01/19 – 12/31/20

Overlap: None

Agency: Children's Cancer Research Fund

Grant number: Team Nat Fund Project-1

Role: Principal Investigator

Title of project: "Exosomal mRNA Signatures to Inform Prognosis of Patients with Osteosarcoma"

Direct Costs:

Period of Award: 09/01/19 – 08/31/20

Hypothesis and Specific Aim: The **hypothesis** that, xenograft models of osteosarcoma will enable discovery of exosome-based biomarkers that predict tumor biological behavior. This project has a single specific aim:

Specific Aim 1 - To use xenograft models of human osteosarcoma with distinct metastatic propensity for exosome biomarker discovery.

Overlap: This project was funded to support continuation of this project through human translation. There is no scientific or budgetary overlap.

Grant assignment: CA190276

Agency: Department of Defense (Congressionally Designated Medical Research Program)

Role on project: Principal Investigator

Title of project: "A Novel Approach Combining Oncolytic Virotherapy and Dual Immune Checkpoint Blockade for Metastatic Osteosarcoma"

Hypothesis and Specific Aims: There are three hypotheses for this project:

Hypothesis 1 is that *systemic treatment with oncolytic VSV will induce osteosarcoma tumor cell killing and antigen exposure.*

Hypothesis 2 is that *concomitant blockade of the CD47 myeloid checkpoint will enhance phagocytosis of tumor cells by antigen-presenting cells.*

Hypothesis 3 is that *the effects of VSV and CD47 blockade will create an inflammatory milieu that promotes activation of tumor-specific T-cells and natural killer cells, enhancing responsiveness to blockade of the PD-1 immune checkpoint and improving outcomes of patients with metastatic osteosarcoma.*

The project has three specific aims.

Specific Aim 1: Optimize the dose and schedule for combination VSV-IFN β -NIS and dual blockade of myeloid and T-cell exhaustion immune checkpoints using P-DIC in orthotopic, syngeneic (immunocompetent) mouse models of metastatic osteosarcoma.

Specific Aim 2: Define the immunological effects and anti-tumor mechanisms of action of VSV-IFN β -NIS and dual blockade of myeloid and T-cell exhaustion immune checkpoints using P-DIC in orthotopic mouse models of metastatic osteosarcoma.

Specific Aim 3: Characterize the safety, efficacy, and immunomodulatory effects of combination therapy using VSV-IFN β -NIS and P-DIC in dogs with naturally occurring metastatic osteosarcoma.

Dates: 07/01/20-06/30/22

Total Direct Costs:

Effort: 0.6 person-calendar months

Overlap: This project seeks to continue development of P-DIC, which was conceived as part of this project, through clinical translation in dogs with spontaneous osteosarcoma as the last step prior to IND and clinical testing in human patients

Agency: AKC Canine Health Foundation

Grant number: CHF Grant 02806-MOU (Competitive Renewal of Grant 02234-MOU)

Principal Investigator: Modiano, Jaime

Title of project: “Prevention of Canine Hemangiosarcoma: Lifetime Follow-Up”

Dates: 08/01/20 - 07/31/24

Total Direct Costs:

Effort: 0.12-person-calendar months

Overlap: None

Agency: Venn Foundation

Grant number: none assigned

Principal Investigator: Modiano, Jaime (with Brenda Weigel and Logan Spector),

Title of project: “Molecular Signatures of Biological Behavior in Osteosarcoma – Technology Accelerator”

Specific Aims:

Specific Aim 1: To generate conserved, exosome-associated mRNA signatures associated with metastatic propensity

Specific Aim 2: To validate the exosome mRNA signatures in an annotated sample cohort from children with osteosarcoma to establish their prognostic value

Dates: 09/01/20 - 08/31/23

Total Direct Costs:

Effort: 0.12-person-calendar months

Overlap: This project was funded to develop a liquid biopsy test from exosomes that provides prognostically significant biomarkers to guide treatment of children with osteosarcoma. There is no scientific or budgetary overlap.

Grant assignment: CA191225

Agency: Department of Defense (Congressionally Designated Medical Research Program)

Principal Investigator: Kim, Jong Hyuk

Role on Project: Mentor/Career Guide

Title of project: “Chromatin Accessibility and the Convergent Oncogenic Pathways of Angiosarcomas”

Dates: 07/01/2020 - 06/30/23

Direct Costs:

Effort: 0.12-person-calendar months

Overlap: None

Grant assignment: 2019-1

Agency: Rein in Sarcoma (RIS) Foundation

Role on Project: Principal Investigator

Title of project: “eBAT as a Modulator of the Myeloid Immune Checkpoint in Cancer”

Dates: 03/01/20 - 02/28/21

Direct Costs:

Effort: 0.12-person-calendar months

Overlap: None

What other organizations were involved as partners?

1. Yale University

New Haven, CT

Collaboration (subaward)

2. Mayo Clinic

Rochester, MN

Collaboration (no funds or subaward)

3. Texas Tech University Health Sciences Center

El Paso, TX

Collaboration (no funds or subaward)

4. Stanford University

Palo Alto (Stanford), CA

Collaboration (no funds or subaward)

8. SPECIAL REPORTING REQUIREMENTS**COLLABORATIVE AWARDS:**

Not applicable

QUAD CHARTS:

Not applicable

9. APPENDICES:**Appendix I**

1. Mills, L. J. et al. Comparative analysis of genome-wide DNA methylation identifies patterns that associate with conserved transcriptional programs in osteosarcoma. *Bone*, 115716, doi:10.1016/j.bone.2020.115716 (2020).

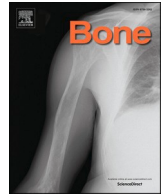
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APPENDIX



Comparative analysis of genome-wide DNA methylation identifies patterns that associate with conserved transcriptional programs in osteosarcoma

Lauren J. Mills^{a,b,*}, Milcah C. Scott^c, Pankti Shah^d, Anne R. Cunanan^d, Archana Deshpande^e, Benjamin Auch^e, Bridget Curtin^h, Kenneth B. Beckman^e, Logan G. Spector^{a,b}, Aaron L. Sarver^{a,f,g}, Subbaya Subramanian^{a,h}, Todd A. Richmond^d, Jaime F. Modiano^{a,f,g,i,j,k}

^a Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA

^b Childhood Cancer Genomics Group, Department of Pediatric, School of Medicine, University of Minnesota, Minneapolis, MN 55455, USA

^c Department of Microbiology and Immunology, Center for Immunology, School of Medicine, University of Minnesota, Minneapolis, MN 55455, USA

^d Roche Sequencing Solution, Pleasanton, CA, USA

^e University of Minnesota Genomics Center, Minneapolis, MN 55455, USA

^f Animal Cancer Care and Research Program, University of Minnesota, St. Paul 55108, USA

^g Institute of Health Informatics, University of Minnesota, Minneapolis, MN 55455, USA

^h Department of Surgery, University of Minnesota, Minneapolis, MN 55455, USA

ⁱ Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, USA

^j Center for Immunology, University of Minnesota, Minneapolis, MN 55455, USA

^k Stem Cell Institute, University of Minnesota, UDS Institute for Engineering in Medicine, Minneapolis, MN 55455, USA

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Keywords:

Osteosarcoma
DNA methylation
EPIGENETIC
Comparative oncology

ABSTRACT

Osteosarcoma is an aggressive tumor of the bone that primarily affects young adults and adolescents. Osteosarcoma is characterized by genomic chaos and heterogeneity. While inactivation of tumor protein p53 (*TP53*) is nearly universal other high frequency mutations or structural variations have not been identified. Despite this genomic heterogeneity, key conserved transcriptional programs associated with survival have been identified across human, canine and induced murine osteosarcoma. The epigenomic landscape, including DNA methylation, plays a key role in establishing transcriptional programs in all cell types. The role of epigenetic dysregulation has been studied in a variety of cancers but has yet to be explored at scale in osteosarcoma. Here we examined genome-wide DNA methylation patterns in 24 human and 44 canine osteosarcoma samples identifying groups of highly correlated DNA methylation marks in human and canine osteosarcoma samples. We also link specific DNA methylation patterns to key transcriptional programs in both human and canine osteosarcoma. Building on previous work, we built a DNA methylation-based measure for the presence and abundance of various immune cell types in osteosarcoma. Finally, we determined that the underlying state of the tumor, and not changes in cell composition, were the main driver of differences in DNA methylation across the human and canine samples.

Significance: Genome wide comparison of DNA methylation patterns in osteosarcoma across two species lays the ground work for the exploration of DNA methylation programs that help establish conserved transcriptional programs in the context of varied mutational landscapes.

1. Introduction

Osteosarcoma is a rare disease, there are fewer than 1000 cases diagnosed in the U.S. each year, mostly in children and adolescents [1]. However, these numbers fail to convey the impact that the disease has on patients, their families, caregivers, and the extended community due

to its significant morbidity and years of life lost. Recent work is starting to increase our fundamental understanding of osteosarcoma [2] but more than half of patients still relapse and die from metastatic disease within 10 years [1,3]. Osteosarcoma has been reported in every vertebrate species [4]. It is as rare in most animals as it is in humans, and when it occurs, it is most common in the axial skeleton [4]. Dogs are a

* Corresponding author at: Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA.

E-mail address: ljmills@umn.edu (L.J. Mills).

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notable exception. Osteosarcoma is extremely common in large and giant dogs, and similar to children the disease occurs most frequently in the appendicular skeleton [4,5].

Many studies have evaluated the genomic landscape of osteosarcoma in humans and in animal models. The heterogeneity of this disease is remarkable, both within and among species [5–10]. Loss of function of the *TP53* gene seems to be a nearly universal event in spontaneous osteosarcoma, thus it might be causally related to the chaotic genomes that are characteristic of this condition [7]. Aside from *TP53* loss of function mutations, recurrent genomic aberrations are rare within species and even more infrequent between species. But unlike its highly heterogeneous mutational landscape, the transcriptional programs that characterize human and canine osteosarcoma are highly conserved [8,11]. More specifically, one key transcriptional program defined subsets with higher (or lower) rates of tumor cell proliferation and turnover, inferred from the expression of gene clusters associated with cell cycle progression, mitosis, DNA damage repair, and chromosomal instability [8,11,12]. We created a method to quantify this expression using a gene cluster expression summary score (GCESS) [8] and showed that this GCESS was inversely associated with overall survival in both dogs and humans. The other salient conserved transcriptional programs defined subsets associated with abundance of immune and inflammatory cells in the microenvironment, inferred from the expression of genes uniquely or predominantly expressed by cells of the innate and adaptive immune system [8]. Curiously, the immune GCESS were only predictive of survival time and metastasis in human patients but not in dogs.

The discrepancy between the highly heterogeneous mutational landscape of human and canine osteosarcomas with diverse putative genetic drives and their relatively homogeneous transcriptional landscape indicates these two sets of events are probably unrelated in both species. In other words, these tumors do not seem to be caused by driver events that activate or repress specific transcription. Instead, it is most likely that osteosarcomas in humans and in dogs are convergent entities where epigenetic controls of gene expression driven by selection ultimately give rise to the limited molecular pathways that achieve the tissue organization required to form osteosarcoma tumors. One such epigenetic control is DNA methylation, which in turn is a major determinant of chromatin accessibility.

For this study, we sought to determine the role of DNA methylation in establishing the conserved transcriptional programs observed in human and canine osteosarcoma. Our data show that, indeed, there are conserved modules of methylation in human and canine osteosarcomas. In spite of the apparent species differences, such conserved processes must reflect pathogenetically significant events that contribute to risk, progression, and therapeutic failure, and understanding their mechanisms will aid the development of better methods to identify risk and prognosticate progression, and ultimately more effective strategies for treatment, control, and eventually prevention.

2. Results

2.1. Genome wide DNA methylation patterns in human and canine osteosarcoma

Our objective was to establish mechanisms that control conserved transcriptional programs in human and canine osteosarcoma tissues. Targeted bisulfite sequencing was used to measure genome wide DNA methylation levels in 24 samples of human osteosarcoma and 44 samples of canine osteosarcoma. In order to focus the comparisons on conserved mechanisms that underlie the cellular and molecular organization of osteosarcoma, we only considered methylation measurements from homologous regions of the two genomes (54 K human and 58 K canine genomic regions, 19% and 33% of total measured regions respectively, Fig. 1A). Weighted gene correlation network analysis (WGCNA) [13,14] clustering was applied to the DNA methylation data to reduce complexity by identifying genomic regions with highly

correlated DNA methylation profiles across the osteosarcoma samples in both species. WGCNA generates large clusters of genomic regions with highly correlated DNA methylation measurements across samples and produces a summary value for each cluster. Clusters were constructed using the same parameters for the human and canine samples and the same correlation cut off value was used to establish cluster membership. We thus condensed 12,165 unique methylation measurements from the human osteosarcoma samples into 43 clusters containing between 16 and 2529 individual methylation measurements (Fig. 1A). Five modules contained less than 100 measurements and 6 modules contained more than 1000. Similarly, we condensed 6099 unique methylation measurements from the canine osteosarcoma samples into 12 clusters containing between 139 and 2532 individual methylation measurements (Fig. 1A). The representative methylation value for each cluster, equivalent to the first principal component eigenvalue, is given in the heatmaps in Fig. 1B and C. For both the human and canine clusters, methylation measurements from multiple chromosomes are clustered together indicating larger methylation programs that extend beyond methylation measurements in local genomic regions. While only methylation measurements from homologous genomic regions were used for clustering, the human and canine samples were clustered independently because we were interested in identifying methylation clusters that contain homologous genomic regions in the two species. The highest levels of region overlap were seen between human cluster ME1 and canine clusters ME1 (31.75%) and ME10 (33.26%), between human cluster ME5 and canine cluster ME3 (33.33%), and between human cluster ME7 and canine cluster ME8 (33.61%) (Supp Table). K-means clustering using the Euclidean distance of the summarized methylation values for each cluster was used to identify clusters that showed similar patterns across either the human or canine osteosarcoma samples, as indicated by the colored toe bars at the bottom of Fig. 1B and C. The color scheme in Fig. 1B and C is maintained throughout the rest of the manuscript and used as a reference for clusters. Some of the clusters share genomic regions across both species, as indicated by shared colors in the toe bars (blue, green, orange), while other clusters are species specific (red, light orange, yellow, purple, and black, Fig. 1B, C, and D). We assigned the nearest gene (≤ 1000 bp) to each methylation measurement and performed pathway analysis using the Reactome database for each methylation cluster. Not all methylation clusters resulted in significant pathway enrichment, but with the exception of the black group, all others had 1 or more individual groups of methylation marks with enriched functional pathways (Fig. 1D and Supp Table). Predictably [13,14], genes in the Reactome pathways “Transcriptional Regulation by TP53: (R-HSA-3700989), along with other pathways associated with signal transduction, were enriched in modules in the blue group, and genes in the “Transcriptional regulation by RUNX2” and “Transcriptional regulation of pluripotent stem cells” pathways were enriched in modules in the green group in samples from both species.

2.2. Association between methylation clusters and osteosarcoma transcriptional programs

Previously, we identified shared transcriptional programs in human and canine osteosarcoma tissues that were associated with proliferation (cell cycle), presumably of the tumor cells that were inversely associated with overall survival in both species [8]. We also showed that the presence of immune cells (immune1 and immune2) in the microenvironment was directly associated with overall survival and time to metastasis, but only in human patients [8]. A subset of the samples used to identify those transcriptional programs were included in this study (human $n = 16$, canine $n = 9$). We calculated correlations between DNA methylation clusters and the summarized gene expression values for the expression groups from Scott et al. [8] using the summarized methylation values for each cluster identified with WGCNA (Fig. 2). In addition, we correlated DNA methylation to the age at diagnosis and overall survival time for the canine samples (Fig. 2B). Fig. 2 shows that the



(caption on next page)

Fig. 1. Genome wide DNA methylation patterns in canine and human osteosarcoma samples. Summary of analysis and the number of genomic regions included in each step (A). DNA methylation values were calculated at homologous genomic regions in canine (B, $n = 44$) and human (C, $n = 24$) primary osteosarcoma tumor samples. Genomic regions were clustered based on correlation in each species resulting in 12 canine clusters and 43 human clusters containing genomic regions that have highly correlated methylation values across all osteosarcoma samples. Heatmaps represent the weighted average DNA methylation value for the cluster in each sample. Red is a high methylation value and blue is a lower methylation value. Pathway analysis was performed using Reactome on the gene nearest (≤ 1000 bp) the underlying genomic region in each DNA methylation cluster. Clusters that share many of the same pathways are color coded the same in B and C and a summary of the pathways is given for each colored block in D. The unknown cluster did not have any significantly enriched pathways. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

methylation clusters not only behave similarly when looking at the global DNA methylation patterns, but also when they are compared to downstream transcriptional programs. Specifically, the methylation clusters in the green group are positively correlated with cell cycle expression and inversely correlated with immune expression in both the dog and the human samples. In contrast the methylation clusters in the orange group, in both species, and the red group, in the human samples, show the opposite behavior and are inversely correlated with expression of cell cycle-associated genes and positively correlated with expression of immune-related genes.

2.3. Immune cell abundance across osteosarcoma samples

The presence or absence of specific immune cell infiltrates is an important predictor of survival in many solid tumors [15] including human osteosarcoma [8]. DNA methylation data, from 450 K arrays, has been shown to be more effective at predicting the abundance of specific cell types in gold standard mixtures than expression data from RNA-seq¹⁶. Building on these previous methods, we obtained whole genome bisulfite sequencing data for 12 pure human cell populations from the BLUEPRINT project [17]. Whole genome bisulfite sequencing (WGBS) data were summarized to the same human/canine homologous regions used as input for WGCNA analysis. Cell populations of interest included 10 immune cell populations as well as osteoclasts and mesenchymal stromal cells (MSC), which were used as surrogates for tumor cells as WGBS data for osteoblasts is not available in the public domain. We performed differential methylation tests between all pairs of pure cell populations, and the top 100 genomic regions with the largest differences in methylation levels were selected from each test and combined (1312 unique regions) to generate a custom signature file in CIBERSORT (SFigure 1). Once generated, this custom signature file accurately predicted the cell type in computationally generated mixtures based on methylation levels (SFigure 2). WGBS data from known mixtures of canine cells were not available to further test predictions from this method, so we relied on the approach used to identify homologous genomic regions to convert canine methylation measurements to their human genome equivalents. We then applied the same signature file to both species and, as expected, MSCs had the highest signal across all samples (Fig. 3A & B). The data also suggests that all other stromal and immune cell types that we interrogated were infrequent (low abundance) in human osteosarcoma samples (Fig. 3A), while suggesting that monocytes, CD8+ T-cells, and osteoclasts were relatively more abundant in canine osteosarcoma samples (Fig. 3B). It has been previously shown that higher levels of CD8+ T-cells in the blood [18] or tumor samples [19] of dogs with OS is a positive prognostic factor for survival. The presence of CD3+ T-cells has been seen in human OS tumors but overall immune infiltrate levels of OS have been estimated to be intermediate when compared to the tumors in TCGA, and lower than other soft tissue sarcomas as well as melanoma and lung cancer where immune checkpoint blockade has clinical benefits [20].

Associations between global DNA methylation modules and predicted immune cell abundance.

Global DNA methylation patterns are highly specific to cell type, though obtained through separate analysis, it is possible that the DNA methylation clusters identified via WGCNA were a result of the differences in the abundance of different cell types in the osteosarcoma samples. Though generated from the same starting regions (Fig. 1A),

there was a small overlap between the regions included in the WGCNA methylation clusters and the custom signature file used with CIBERSORT (352/12,165 from the human data and 147/6099 from the canine data). To understand if any of our discovered methylation clusters were indirectly measuring immune cell abundance or the state of the tumor tissue, we calculated correlations between the summarized methylation values for each methylation cluster and predicted immune cell abundance scores (Fig. 4). In both species, similar to the relationships between the methylation scores and transcription measures (Fig. 2), methylation clusters of the same color tend to have similar relationships to the cell abundance measures (Fig. 4). In the human data most of the methylation clusters showed similar, positive, correlations to MSC while they displayed a more varied behavior overall (Fig. 1A) and against key transcriptional patterns (Fig. 2A). Interestingly, the orange and green clusters both have positive correlations to MSC abundance while they have opposite correlations, from each other, with the immune and cell cycle expression measures (Fig. 2). In the canine data strong positive and negative correlations are seen between MSC, CD8+ T-cells and inflammatory macrophages. Also, in the canine data the opposite correlations are seen between the green and orange/light orange groups mirroring what is seen when compared to the transcriptional patterns (Fig. 2B). In the human data, immune cell abundance is very low and the strongest correlations are seen between the methylation modules and the MSC abundance measures. While a few of the methylation clusters may reflect the abundance of immune cells, many more seem to reflect the underlying states of the tumor itself. In the canine data, immune cell abundance is higher and more varied, and the same modules show strong correlations to both transcriptional measures of immune cell infiltration (Fig. 2B) and methylation-based measures (Fig. 4B), so more of the original methylation clusters (green clusters) may be reflective of cell abundance. But many clusters do not show a strong correlation to any cell abundance measure and most likely reflect other aspects of the underlying tumor state.

3. Discussion

The molecular basis of osteosarcoma has received considerable attention during the last decade [21–29]. Several genetic mutations including *TP53*, *RB*, *MYC*, and *RUNX2* have been strongly implicated in the development of osteosarcoma [7,27,30–41] but our understanding of osteosarcoma epigenome is still limited [8–43]. The clinical outcomes continue to be dismal and have landed this disease among the “most wanted” for development of new, effective therapies. Elements of our failure to understand osteosarcoma pathobiology and treatment include tumor heterogeneity, a lack of robust prognostic factors, and the fact that current therapies fail to prevent relapse and/or metastasis for many human patients and most canine patients. These challenges are also compounded by the *orphan disease* status of osteosarcoma.

Recently, we showed that transcriptional programs in human and canine osteosarcoma defined the proliferation and immune signature in the tumor microenvironment that are associated with aggressiveness and outcomes in osteosarcoma. Notably, our studies and other genomic studies have failed to identify recurrent translocation or mutational profiles associated with the transcriptional programs in human and canine osteosarcoma. In the absence of recurrent mutations the aberrant expression of pro-survival and metastatic genes noticed in osteosarcoma may be in part due to deregulation of microRNAs [42,43]. In addition to

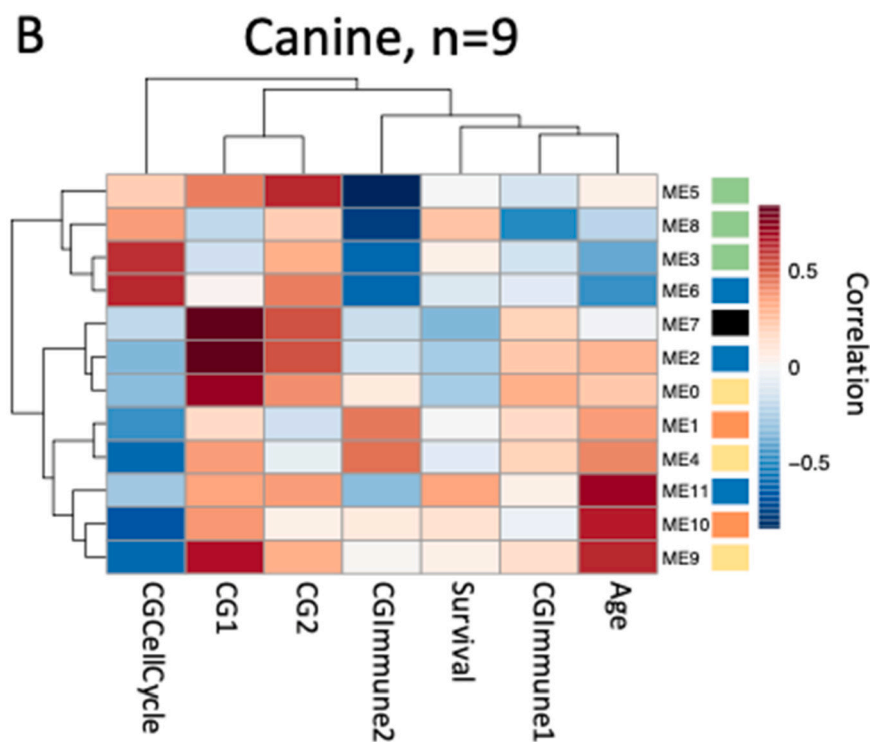
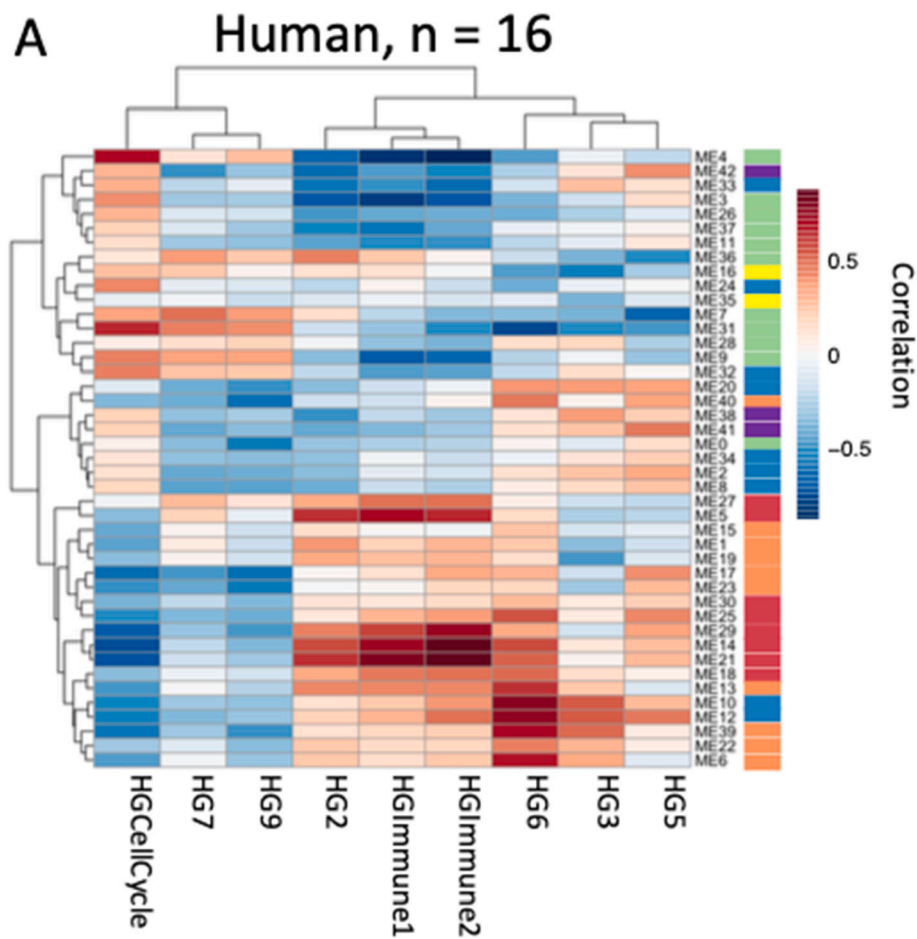


Fig. 2. Correlation between methylation clusters and important expression programs in osteosarcoma. Correlations between methylation summary values for each DNA methylation cluster and previously described expression programs were calculated for canine (A, $n = 9$) and human (B, $n = 16$) samples where methylation and expression data was available. Correlations were also calculated for additional phenotypic data for canine samples. Heatmaps indicate Pearson correlation values between each DNA methylation cluster and phenotype where red is strong positive correlation and blue is strong negative correlation. Color bars next to module names indicate pathway analysis results and are the same as in the previous fig. HG = human GCESS, CG = canine GCESS from Scott et al. 2018 [8]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

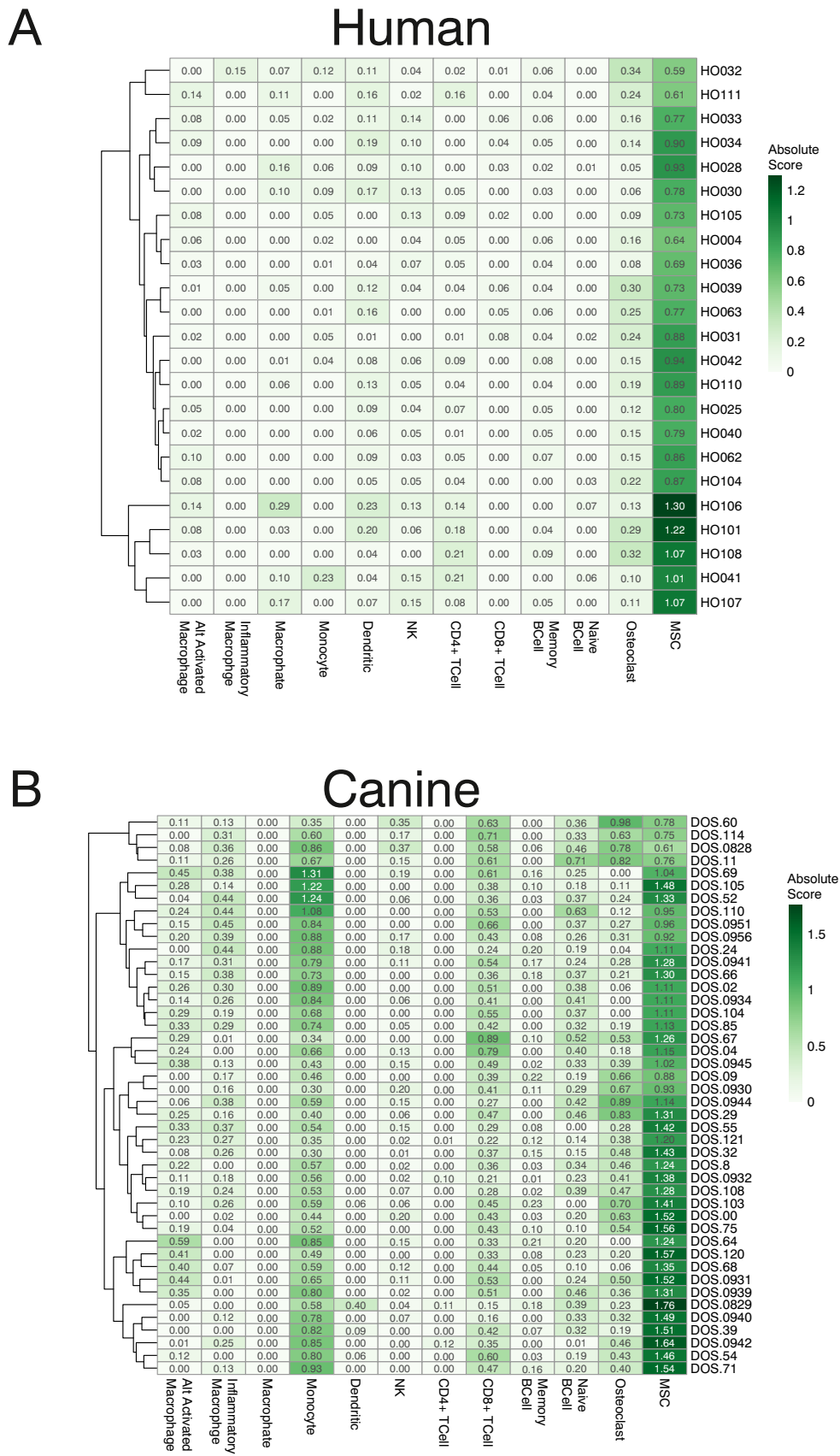


Fig. 3. Immune cell abundance across osteosarcoma samples. WGBS data from pure human immune cell populations and mesenchymal stromal cells were obtained from the BLUEPRINT project. DNA methylation values were summarized to match those measured in our osteosarcoma samples and used to build a model to distinguish between each cell type. This model was used with CIBERSORT to measure the absolute cell abundances for each cell type in all of our osteosarcoma samples. Heatmap color and value in each cell indicate the abundance of each cell type in each sample for canine (A) and human (B) osteosarcoma tumors. Absolute mode was used to generate cell abundance scores and are not relative so columns will not sum to 1 (100%).

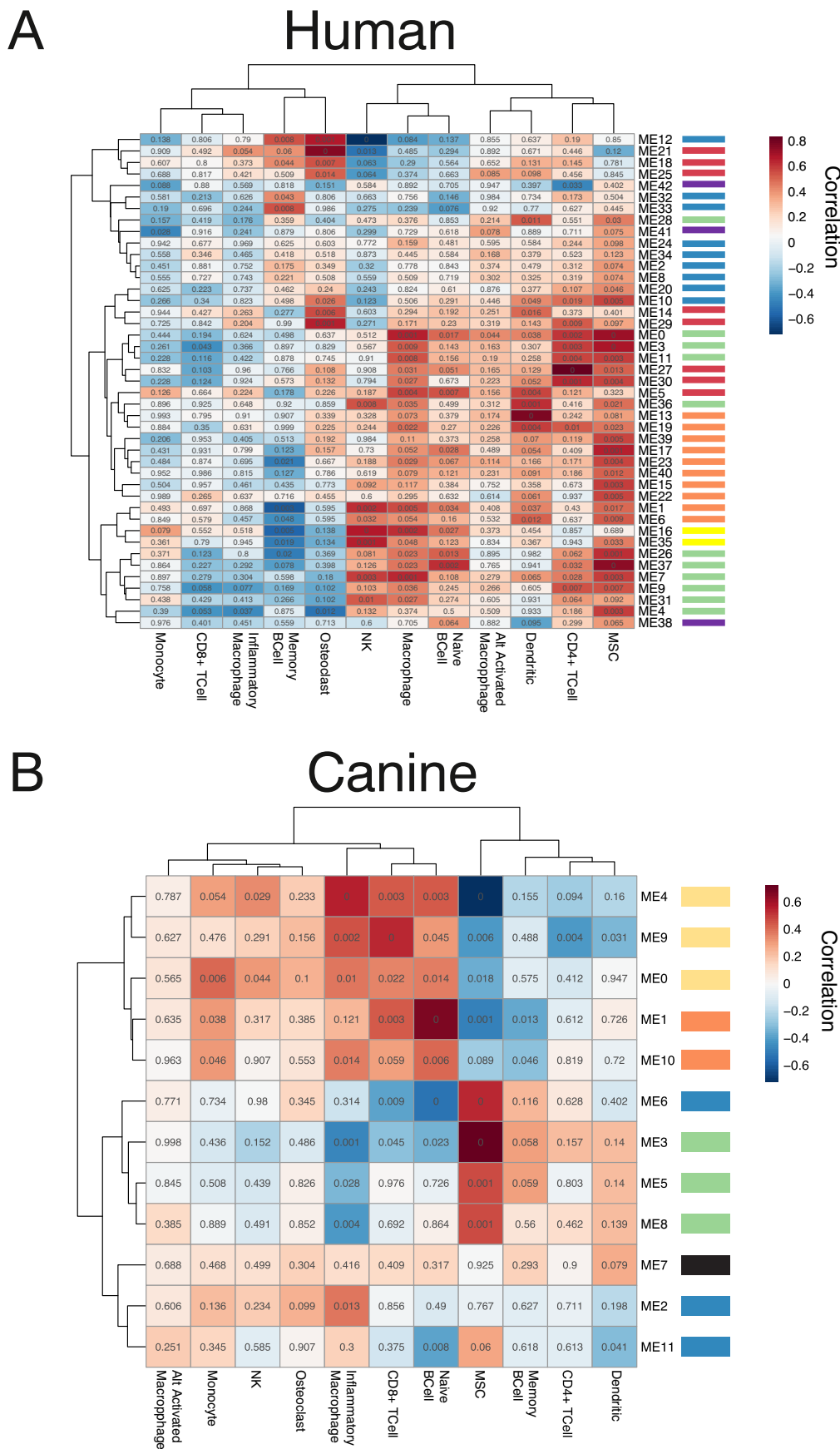


Fig. 4. Correlation between methylation clusters and cell type abundances in OS. Correlations between methylation summary values for each DNA methylation cluster and CIBERSORT absolute cell type abundances were calculated for canine (A, $n = 44$) and human (B, $n = 24$) samples. Heatmaps indicate Pearson correlation values between DNA methylation cluster and cell type abundance where red is a strong positive correlation and blue is a strong negative correlation. Values in each cell are the Student asymptotic p -value of the given correlation based on the number of samples. Color bars next to module names indicate pathway analysis results and are the same as in the previous figures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

microRNAs the transcriptional programs may be regulated by epigenetic alterations [44–46]. Using genome-wide methylation profiles of human and canine osteosarcoma, here we show that transcriptional programs associated with cell proliferation and immune signature are associated with methylation patterns in both the species. Specifically, DNA methylation across the blue, green and orange clusters (Fig. 1) encompassing 1773 genomic locations behave similarly between human and canine while other regions are species specific. Correlative analysis of genome-wide methylation patterns and corresponding transcriptional profiles in human and canine osteosarcoma revealed strong correlations between key transcriptional patterns and DNA methylation measurements. For example, conserved green methylation clusters were positively correlated with cell cycle expression programs in both species while conserved orange methylation clusters were negatively correlated again in both species. These same methylation clusters displayed the opposite behavior when compared to the immune expression profiles highlighting the tradeoff between tumors with high proliferation and those with higher immune components also seen in Scott et al. [8]. These findings have implication for developing potential biomarkers or other predictive measures for identifying tumors that might be more aggressive due to higher rates of proliferation or to track very low levels of immune cell infiltration that is often missed using standard pathology methods.

Next, we generated a new algorithm based on CIBERSORT and past use of Illumina 450 K methylation profiles [16] to predict immune cell abundances from whole genome bisulfite sequencing data. Our analysis shows a well-defined difference between immune cell profiles among human and canine osteosarcoma samples. Canine osteosarcoma shows much higher presence of immune cells across the samples analyzed, average absolute abundance measures were higher for 6 out of 10 immune cells in the canine samples as well as for osteoclasts and MSC. Notably, we observed increased CD8⁺ T cell signature in canines (mean absolute abundance 0.44) that are relatively less abundant in human samples (mean absolute abundance 0.01). This observation is intriguing because even with higher immune cell infiltration in canine samples, this did not translate to increased survival in canines. Even though canine tumors are primarily driven by the proliferation signatures that are correlated with methylation patterns, we speculate that progression in canine tumors is predominantly driven by cell proliferation with immune cells potentially having less influence in the absence of immunomodulatory therapies.

We also observed high correlations between the global methylation patterns and immune cell abundance in both human and canine osteosarcoma. Strong correlations between single cell types, or cell types with correlated abundances, and methylation modules were not observed leading us to believe that global methylation patterns do not seem to be driven only by the abundance of immune cells in the microenvironment, but most likely reflect the proliferative and pro-survival status of the tumor cells. Inhibiting pro-survival pathways linked to osteosarcoma progression has therapeutic relevance. Conventional therapies—including the DNA-intercalating drugs doxorubicin and cisplatin [47] and methotrexate, an anti-metabolite in combination with leucovorin—have very serious side effects, including decreased production of blood cells that leads to infection and damage to the bladder and kidney. Platinum-containing drugs often cause hearing loss. New drugs such as muramyl tripeptide [48], rapamycin inhibitor [49], and Trastuzumab [50], show only marginal increases in overall survival, leaving survival rates still distressingly low. The dysregulated transcriptional programs that are coupled with methylation patterns can be restored by DNA- and chromatin-modifying drugs 5-Aza (5-Aza-2'-deoxycytidine (5-Aza or decitabine, a hypomethylating agent) and SAHA (Suberanilohydroxamic acid or vorinostat, a histone deacetylase inhibitor). Recently we showed that 5-AZA and SAHA treatment alter the transcriptional landscape of osteosarcoma cells towards one resembling RB expression [45]. Preclinical and early clinical studies combining 5-Aza with chemotherapies, peptide vaccines and immune checkpoint

therapies found evidence that this treatment increases tumor suppressor expression and chemosensitivity. Numerous clinical studies have also reported that 5-aza improves the efficacy of antigen-directed immunotherapy in pediatric sarcomas [48]. Moreover, treating osteosarcoma cell lines with a combination 5-AZA and SAHA induced apoptosis, even in aggressive cell lines (MG63, HOS and 143B) that are typically more resistant to treatment [51].

In summary, we have used genome-wide methylation patterns to reveal association between methylation clusters and conserved transcriptional programs of human and canine osteosarcoma. This first large-scale genome-wide DNA methylation study in both human and canine osteosarcoma revealed specific DNA methylation programs that are highly correlated to gene expression programs important to disease progression and survival in both human and canine osteosarcoma. Further, exploring the global DNA methylation patterns between different cell types we found that the stromal and immune cell types were in low abundance in human osteosarcomas while canine osteosarcoma samples showed relatively greater abundance of monocytes, T cells, and osteoclasts. Genome wide DNA methylation profiling of osteosarcoma samples is rare in both human and canine disease and obtaining DNA from these samples is notoriously difficult. Also, while DNA methylation profiles exist for a large number of human immune cells and subtypes the same data is not available for dogs. These comparative studies on mechanisms that regulate conserved transcriptional programs in both human and canine osteosarcoma are critical to develop biomarkers [52] and therapeutic targets and motives further large scale collection of osteosarcoma samples to include DNA methylation profiling.

4. Methods

4.1. Biospecimen collection and processing

Tumor tissue biospecimens were collected from newly diagnosed human patients or dogs with a confirmed diagnosis of appendicular osteosarcoma prior to treatment with cytotoxic chemotherapy drugs [8,11].

Human specimens ($n = 24$) were obtained from the University of Minnesota Biological Materials Procurement Network (UMN BioNet) under oversight of the University of Minnesota's Institutional Review Board (IRB) with an Exemption-4 category, or from the Cooperative Human Tissue Network (CHTN), also with an IRB Exemption-4. Samples were de-identified and only a limited amount of patient information was provided. Sample collection was done using standardized protocols with a portion of the diagnostic biopsy, obtained as part of a medically necessary procedure flash frozen immediately in liquid nitrogen and stored at -86°C until they were assigned to the project.

Canine specimens ($n = 44$) were obtained from dogs with naturally occurring primary appendicular tumors, recruited between 1999 and 2016. Tumor tissue samples were obtained from tissue biopsy or amputation surgeries that were part of standard treatment protocols and with owner consent under supervision by the appropriate Institutional Animal Care and Use Committees (University of Minnesota protocol numbers 0802A27363, 1101A94713, 1312-31131A) or the University of Colorado Institutional Review Board or Institutional Animal Care and Use Committee (AMC 635040202, AMC 200201jm, AMC 2002141jm, 02905603(01)1F, COMIRB 06-1008). Some of these canine tumor samples were flash frozen immediately in liquid nitrogen and stored at -86°C until they were assigned to the project; others were immediately placed in complete, sterile cell culture media consisting of Dulbecco's Modified Eagle Media supplemented with 10% fetal bovine serum, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 100 $\mu\text{g}/\text{mL}$ Primocin and transported to the lab by overnight courier at 4°C , and flash frozen at -86°C after examination and processing upon arrival. Transport did not have a meaningful effect on tumor viability based on gross examination and on the functional capability to establish

viable, immortalized osteosarcoma cell lines from the samples.

After collection, human and canine tumor tissues were removed from -86°C and sectioned to avoid areas of necrosis. Thirty milligrams of tissue were placed in 80 μL of phosphate buffered saline (PBS) solution and pulverized in a tissue homogenizer. Isolation of genomic DNA was done according to the manufacturer's protocol using the QIAamp DNA Mini Kit from Qiagen.

4.2. Bisulfite conversion, library preparation and target region capture

Illumina library preparation, bisulfite conversion and bead capture was performed as specified in the Roche SeqCap Epi protocol outlined in Li et al. [53]. Probes for the canine version of the SeqCap Epi were designed for canFam3.1 CpG islands as specified by the UCSC genome browser and CpG islands identified using EMBOS cpGREP (<http://www.bioinformatics.nl/cgi-bin/emboss/cpgreport>) that were homologous to hg19. An average of 48 and 46 million 2×125 paired end Illumina reads were generated for the human and canine samples.

4.3. Bisulfite-seq data analysis

Illumina specific adapter sequences and low quality sequences were removed from raw sequencing data using Trimmomatic [54]. Reads were aligned to a bisulfite converted human (hg19) or canine (canfam3.1) genome using WALT [55]. Duplicate sequences were removed from alignment data using MethPipe [56] duplicate-remover and bisulfite conversion rates were estimated for each sample using bsrates. All samples had conversion efficiencies $>99\%$. Sequencing depths for each sample were such that an average of 88.3% and 81.3% of targeted CpGs were sequenced at a $10\times$ depth or higher. MethPipe methcounts was used to calculate methylation levels and read coverage at individual CpGs in each sample. A custom perl script was used to isolate CpGs with a read depth of 10 or greater and MethPipe roimethstats was used to summarize methylation levels in 500 bp windows, generated by BEDtools [57] windowBed, along the capture targets in each genome. CpG methylation levels were expressed as a value between 0 and 1.

4.4. Identification of homologous genomic regions and region to gene mapping

University of California Santa Cruz (UCSC) utility liftOver with the appropriate genome wide alignment file was used to convert the canine genomic coordinates for each 500 bp window from CanFam3.1 to hg19. and the human genomic coordinates for each 500 bp window to GRCh38. Converting both sets of coordinates to GRCh38 resulted in the highest number of 500 bp windows being conserved between the two species. Genomic regions were considered homologs if the converted GRCh38 coordinates were within 1,000 bp of each other as calculated by BEDtools closest.

4.5. Identification of highly correlated DNA methylation patterns with WGCNA, cluster membership and correlation analysis

WGCNA analysis was carried out via a custom R script. Methylation levels for homologous genomic regions were the starting input for WGCNA in each species. All homologous genomic regions with a variance greater than 0 were included in the WGCNA analysis. Soft thresholding power was calculated for each species separately using pickSoftThreshold (human power = 10, canine power = 14, sup figure). Clusters (aka modules) were generated using blockwiseModules with the following parameters for both species and the species-specific power value.

```
blockwiseModules(<inputMethmatrix>,
  maxBlockSize = 1500,
  power = 14|10,
  TOMType = "signed",
```

```
corType = "bicor",
  minModuleSize = 20,
  mergeCutHeight = 0.25,
  verbose = 3,
  numericLabels = T,
  saveTOMs=T,
  saveTOMFileBase=<name>)
```

Summary methylation values for each cluster were obtained from the MEs element of the output from blockwiseModules. The correlation between the methylation level at each genomic region to every methylation cluster summary value was calculated using signedKME and a genomic region was determined to be a member of a specific cluster if the signed module eigengene based connectivity measure (KME) was ≥ 0.7 . This classification allows individual genomic regions to be assigned to multiple clusters. Pearson correlations between summary methylation values for each cluster and other phenotypic measures (gene expression, age, survival time, immune cell abundances) were calculated using the WGCNA function cor and Student asymptotic p -values were calculated for each correlation value using corPvalueStudent and the number of samples for the multiple sample correction. Survival was defined as time to death (humans) or time to death or euthanasia (dogs). Identical WGCNA analysis performed on a randomized human dataset resulted in the generation of 6 clusters that contained 113 DNA methylation regions and randomized canine data did not produce any clusters.

4.6. Generation of custom signature file for CIBERSORT

BigWig files containing genome wide methylation levels from WGBS data for multiple replicates of 12 cell types were downloaded from the BLUEPRINT epigenome project. BigWig files were converted to bed-Graph using the UCSC utility bigWigToBedGraph, sorted using BEDtools sort and mean methylation levels were calculated for the same 500 bp windows used for the WGCNA analysis using BEDtools map -c 4 -o mean. These summarized methylation levels were used as the input for pairwise differential methylation tests between every cell type, 66 tests in all. Differential tests were performed using the limma [58] package in R. The top 100 most differentially methylated regions, based on adjusted p -value, from all differential tests were isolated and the raw methylation values for each cell type for these regions were combined and used as input to CIBERSORT to generate the custom signature file. Raw methylation values for these same regions for all osteosarcoma samples were used as the mixture file input for CIBERSORT. Absolute Mode was used to quantify cell abundance to try and account for cell types that may be missing from our model but are in the tumor.

4.7. Relevant gene names

TP53 – Tumor Protein 53
 RUNX2 – Runt-related transcription factor 2
 RB1 – RB Transcriptional Corepressor 1
 MYC – MYC Proto-Oncogene, BHLH Transcription Factor

4.8. Data access and sharing

All raw sequencing data, DNA methylation measurements, cluster membership and DNA methylation summary values have been submitted to GEO GSE149679

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CRedit authorship contribution statement

LM – analyzed the data, developed the story, wrote first draft of results, outlined intro and discussion.

MS – worked with Roche Sequencing Solutions-Madison (RSS-Madison) to establish approach for methylation analysis and develop kits. Intimately involved with design and initial data analysis. Prepared all canine samples for the project.

PS – RSS-Madison. Performed the work to develop and validate the methylation kits and completed QC of the data. Presented original poster that provided a template for analysis.

AC – Roche Diagnostics. Facilitated in the provision of sequence capture products, protocols and training requirements.

AD – UMGC. Involved in design of canine methylome kit. Provided oversight for methylation sequencing.

BA – UMGC. Involved in design of canine methylome kit. Performed technical aspects of bisulfite sequencing.

BC – prepared all human samples for the project.

KB – UMGC. Involved in design of canine methylome kit. Provided funding and oversight for the project.

LS – Sobiech group. Provided funding and helped with intellectual conceptualization of the project.

AS – Sobiech group. Provided funding and helped with intellectual conceptualization of the project.

SS – Sobiech group. Provided funding and helped with intellectual conceptualization of the project.

TR – Roche Sequencing Solutions. Designed canine methylome approach and kit (mapped, developed baits, etc.) Provided funding for the project.

JM – Sobiech group. Provided funding and helped with intellectual conceptualization of the project. Overall responsibility for coordinating and moving the project forward.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bone.2020.115716>.

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Tumor secreted extracellular vesicles regulate T-cell costimulation and can be manipulated to induce tumor specific T-cell responses

Xianda Zhao¹, Ce Yuan¹, Dechen Wangmo¹, and Subbaya Subramanian^{1,2,3,4*}

¹Department of Surgery, University of Minnesota Medical School, Minneapolis, MN, 55455, USA

²Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA

³Center for Immunology, University of Minnesota, Minneapolis, MN 55455, USA

⁴Lead Contact

Running title: Tumor secreted extracellular vesicles regulate T-cell costimulation

Correspondence

Subbaya Subramanian, MS, PhD
Department of Surgery
University of Minnesota Medical School
11-212 Moos Tower
Mayo Mail Code 195
420 Delaware Street SE
Minneapolis, MN 55455
Tel: 612-626-4330
Email: subree@umn.edu

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SUMMARY

Tumor intrinsic factors negatively regulate tumor immune cell infiltration and function. Deciphering the underlying mechanisms is critical to improving immunotherapy in cancers. Our analyses of human colorectal cancer (CRC) immune profiles and tumor-immune cell interactions revealed that tumor cell secreted extracellular vesicles (TEVs) induced immunosuppression in CRC. Specifically, TEVs containing microRNA miR-424 suppressed the CD28-CD80/86 costimulatory pathway in tumor infiltrating T cells and dendritic cells, leading to immune checkpoint blockade therapies (ICBT) resistance. Modified TEVs with miR-424 knocked down enhanced T-cell mediated antitumor immune response in CRC tumor models and increased the response ICBT. Intravenous injections of modified TEVs induced tumor antigen specific immune responses. Moreover, injections of modified TEVs boosted the ICBT efficacy in CRC models that mimic aggressively progressing late-stage disease. Collectively, we demonstrate a critical role for TEVs in antitumor immune regulation and immunotherapy response, which could be developed as a novel treatment for ICBT resistant CRC.

INTRODUCTION

Therapies targeting immune checkpoint pathways including the cytotoxic T lymphocyte-associated protein (CTLA-4) and the programmed cell death-1 (PD-1)/PD-1 ligand-1 (PD-L1), have revolutionized cancer treatment (Darvin et al., 2018; Hargadon et al., 2018; Sharma and Allison, 2020). For colorectal cancer (CRC), immune checkpoint blockade therapy (ICBT) is effective in 30-60% of the microsatellite instable-High (MSI-H) subtype (Oliveira et al., 2019). Unfortunately, most CRC patients (>85%) have microsatellite stable (MSS) tumors (Kim et al., 2016; Mlecnik et al., 2016), that do not respond to ICBT. The MSI-H tumors are generally associated with a higher mutational load and are considered immunogenic (Salem et al., 2018; Zhao et al., 2018). Currently, the MSI/MSS phenotype is considered as a biomarker for ICBT response (Le et al., 2017; Mandal et al., 2019). However, this MSI/MSS based stratification alone cannot adequately explain the observed difference in treatment response since immune cell infiltration is also observed in a large proportion of MSS tumors (Mlecnik et al., 2016; Pages et al., 2018). A mechanistic understanding of why the existing tumor infiltrating immune cells are not functionally rescued by ICBT is critical for improving outcomes in both MSS- and MSI- CRC patients.

Intercellular communications between tumor cells and immune cells in the tumor microenvironment (TME) affect both the antitumor immune response and the tumor response to ICBT. One way tumor cells can affect surrounding cells is by secreting extracellular vesicles (EVs) (Kalluri and LeBleu, 2020) and tumor cell-derived EVs (TEVs) can be either immunogenic or immunosuppressive depending on their cargo. For example, PD-L1 in TEVs has strong immunosuppressive effects (Chen et al., 2018; Poggio et al., 2019), while tumor antigens and other immune stimulating factors carried in EVs can induce immune responses (Diamond et al., 2018; Horrevorts et al., 2019; Wolfers et al., 2001). Investigation of additional tumor intrinsic mechanisms and immunosuppressive components in EVs that incite ICBT failures are highly warranted.

It has been demonstrated that CD28-CD80/86 costimulatory signals are critical for ICBT response (Hui et al., 2017; Kamphorst et al., 2017). For example, PD-1 recruits the Shp2 phosphatase, which preferentially inhibits CD28 signaling (Hui et al., 2017). This suggests that anti-PD-1 efficacy is, in part, due to the rescuing of CD28-CD80/86 signaling. Another study demonstrated that CD28-CD80/86 signaling in tumor infiltrating CD8⁺ T cells is essential for the anti-PD-1 response (Kamphorst et al., 2017). Markedly, CD80 expression on antigen presenting cells (APCs) was recognized as a novel mechanism that restricts PD-1 signaling on interacting T cells by forming cis-heterodimers with PD-L1 (Sugiura et al., 2019). This CD80-PD-L1 interaction inhibits CD80 signaling through CTLA-4 thus attenuating the immunosuppression mediated by

CTLA-4(Zhao et al., 2019). These reports led us to ask if other mechanisms, particularly tumor-intrinsic ones, led to CD28-CD80/86 signal dysregulation. We found that TEVs were involved in the disruption of this signaling axis in human CRC patients. Additionally, we investigated whether depleting potential CD28-CD80/86 suppressive factors in TEVs would enhance their immunogenic effects, thus improving antitumor immunity.

RESULTS

Costimulatory molecules CD28, CD80, and CD86 are downregulated on human CRC infiltrating immune cells

We determined the overall adaptive antitumor immune response in human CRC tissues (23 MSI subtype and 45 MSS subtype) by examining T-cell infiltration in the TME. Tumors with well- and poorly- infiltrated T cells were observed in both microsatellite instable (MSI) and microsatellite stable (MSS) subtypes (Figure 1A). Overall, the MSI-CRC had significantly higher numbers of tumor infiltrating T cells compared to MSS-CRC (Figure 1A). This observation corroborated our immune signature analysis of the TCGA-CRC dataset (Figure S1A) and previous reports in CRC(Becht et al., 2016; Llosa et al., 2015; Mlecnik et al., 2016).

Next, we measured immune checkpoint gene (ICG) expression in CRC. Genes expression analysis of a set of 50 ICGs, including CD28, CD80, and CD86, in the TCGA-CRC dataset stratified tumors into ICG- high and -low expression groups. Both MSI- and MSS-CRC tumors were seen in each group (Figure 1B). At the protein level, we measured CD28-CD80/86 costimulatory molecules on human CRC infiltrating T cells and dendritic cells (DCs). Flow cytometry analysis of a cohort of 10 primary human CRC fresh tissues showed that CD4⁺ T cells generally had higher levels of CD28 than CD8⁺ T cells (Figures 1C & S1B). However, variable levels of CD28 expression were observed on both CD4⁺ and CD8⁺ T cells (Figure 1C). Likewise, tumor infiltrating DCs had variable levels of CD80/86 (Figure 1C). These data, though, are composites of TILs and DCs throughout the entirety of the tumor, and do not account for potential spatial differences. To specifically study the CD28-CD80/86 costimulatory molecules on T cells and DCs localized in the center of tumors, we collected 68 archived human CRC tissues (23 MSI subtype and 45 MSS subtype) for immunofluorescence analysis. Only T cells and DCs that intercalated within or between the epithelial component of the CRC/normal colon tissue were selected for scoring (Figures 1D & S1C-D). No/low expression of CD28 (score<0.5, Figure S1C) on T cells was observed in ~60% of all cases in both MSI- and MSS- CRC tumors (Figure 1D). Similarly, DCs located in the central tumor showed no/low expression of CD80/86 (score<0.5, Figure S1C) in ~50%/~33% of all cases (score<0.5, Figure 1D). Taken together, our data showed that the expression of CD28 and

CD80/86 is highly variable and can be weak or absent on tumor infiltrating T cells and DCs in both MSI- and MSS-CRC tumors.

CD28-CD80/86 costimulatory pathway is necessary for ICBT response

Having observed weak/no expression of CD28-CD80/86 on tumor infiltrating immune cells in human CRC, we tested whether loss of CD28-CD80/86 expression affected tumor response to ICBT in a subcutaneous mouse model of CRC. Our data showed that a combination of anti-PD-1 and anti-CTLA-4 could significantly suppress growth of early stage (100-200mm³) subcutaneous tumors (MC38 mouse CRC cell line with MSI phenotype) (Figure 1E). Single drug treatment with either anti-PD-1 or anti-CTLA-4 was found to be less effective than combination treatments (data not shown). However, in both *Cd28*^{-/-} and *Cd80/86*^{-/-} mice, the combination treatment did not suppress growth (Figure 1E & S1E). In human CRC, loss of CD28 and CD80/86 expression occurs in response to a tumor-dependent mechanism, rather than a genetic deficiency. To model this, we administered anti-CD80 and anti-CD86 blocking antibodies to wild type mice bearing early stage tumors to mimic acquired deficiency (depletion after tumor growth). Notably, blocking CD80 after the initial tumor development abolished the efficacy of ICBT (Figure 1E & S1E). Blocking CD86 showed a similar but weaker effect than the CD80 (Figure 1E & S1E). Reanalysis of published data(Prat et al., 2017) indicated that tumors responding to immune checkpoint inhibitors have higher levels of CD28 and CD80/86 expression (Figure S1F). These results suggested that adequate CD28 and CD80/86 expression were necessary for effective ICBT and these findings are consistent with previous studies(Hui et al., 2017; Kamphorst et al., 2017).

MicroRNA-424 is overexpressed in human CRC and inhibits CD28 and CD80 expression

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression(Bracken et al., 2016). To determine if miRNAs regulate CD28-CD80/86 costimulatory molecules in human CRC, we first identified miRNAs that target CD28 and CD80/86 by target prediction (Figure 2A). TCGA-CRC data revealed that miR-424 is upregulated in CRC but downregulated in normal colon tissues (Figure 2A-B). By *in situ* hybridization (ISH), we validated that miR-424 is expressed in cancer tissue, but not in normal colon tissue (Figure 2C and Figure S2A). In another cohort of 21 human CRC and patient-matched normal tissues, we quantified miR-424 expression by qRT-PCR (Figure 2D). Fourteen out of 21 CRC cases showed at least 2-fold upregulation of miR-424 compared to corresponding normal colon tissues (Figure 2D). Hence, miR-424, that potentially inhibits CD28 and CD80 expression, appears upregulated in human CRC.

We next sought to confirm the inhibitory effects of miR-424 on CD28 and CD80. In dual luciferase reporter vectors, we inserted the 3'UTR sequences of CD28 or CD80 (Figures 2E and S2B).

Reporter assays showed that miR-424 significantly reduced the luciferase signal (Figures 2E and S2B). In the reciprocal experiments, site-directed mutations on the 3'UTRs nullified the effects of miR-424 (Figure 2E, Figure S2B). We then isolated primary human T cells and DCs that endogenously expressed CD28 or CD80 from healthy donors' blood and transfected them with miR-424 or scrambled miRs (Figure 2F). Again, miR-424 showed a strong inhibitory effect on CD28 and CD80 protein expression (Figure 2F). These data confirm that miR-424 binds to 3'UTRs of CD28 and CD80 and suppress their expression on T cells and DCs, respectively.

Subsequently, we extended our investigations to mouse CRC models established by subcutaneous injections of CT26 (MSS phenotype) and MC38 (MSI phenotype) mouse CRC cell lines. We assessed the immune cell landscape and miR-424 expression changes during different stages of tumor growth (100-200mm³, 500-700mm³, and 1000-1200mm³). Notably, we observed a gradient decrease of tumor infiltrating immune cell frequencies and CD28 and CD80/86 expression during tumor development (Figures 2G and S2C-E). In contrast, miR-424 expression significantly increased with tumor growth in both CT26 and MC38 tumors (Figures 2G and S2C-E). These findings indicate that increased miR-424 expression is correlated with low CD28 and CD80 protein expression and an immunosuppressive phenotype during tumor growth.

TEVs transfer miR-424 from human CRC cells to tumor infiltrating T cells and DCs

As our data indicated that miR-424 upregulation in human CRC was a potent inhibitor of CD28 and CD80 expression, we sought to identify the mechanism that led to the accumulation of miR-424 in human CRC and tumor infiltrating T cells and DCs. Strikingly, the level of miR-424 was significantly higher in human and mouse CRC cell lines and primary human CRC tumor organoids, than to the species matched naïve T cells and DCs (Figure 3A-B). Extracellular vesicles (EVs) are critical in mediating the transfer of microRNAs and intercellular communication in the TME (Huber et al., 2018; Kalluri and LeBleu, 2020; Valadi et al., 2007). Therefore, we investigated whether tumor cell expressed miR-424 could be transferred to T cells and DCs, leading to strong functional effects in the recipient cells. TEVs enriched for exosomes (average size ~130nm) from human and mouse CRC cell lines were isolated and characterized (Figure S3A-C). In these TEVs, we observed high levels of miR-424 by absolute qRT-PCR (Figure 3A). Using confocal microscopy, we confirmed uptake of fluorescence labeled TEVs by T cells and DCs cells *in vitro* (Figure 3C). We then examined TEVs mediated miR-424 transfer, in a transwell coculture system that contained tumor cells and T cells or DCs. Notably, the presence of tumor cells significantly increased the miR-424 level in T cells and DCs (Figure 3D). Treatment with the sphingomyelinase inhibitor GW4869 was used to block TEVs production without affecting tumor cell viability (Figure S3D). When TEVs

production was blocked by GW4869, tumor cells failed to cause an increase in miR-424 levels in cocultured T cells and DCs (Figure 3D).

We extended our *in vitro* work to mouse models to validate that TEVs are responsible for transferring miR-424 from tumor cells to T cells and DCs in physiologically relevant conditions. WT CT26 cells or CT26 cells expressing CD63-GFP (a marker to trace EVs) were used to establish *in vivo* tumors. The GFP signal was detected in tumor (CT26-CD63-GFP) infiltrating T cells and DCs (Figure 3E). However, T cells and DCs in the tumor draining lymph nodes (TdLNs) and spleen showed no/weak GFP signal (Figures 3E and S3F). T cells and DCs sorted from mice implanted with WT tumors by flow cytometry showed higher levels of miR-424 than T cells and DCs isolated from mice implanted with miR-424 knockout (miR-424KO) tumors and naïve mice spleen (Figure 3F). Further, we determined that activation of T cells and DCs did not regulate their endogenous miR-424 expression (Figure S3G). Thus, the accumulation of miR-424 in tumor infiltrating T cells and DCs is due to the uptake of miR-424 containing TEVs.

Next, we determined the impact of TEVs with/without functional miR-424 on the CD28-CD80/86 costimulatory pathway. We first characterized TEVs isolated from WT tumor cells, miR-424 inhibitor (miR-424 antisense oligonucleotides) transfected tumor cells, and miR-424KO tumor cells. We confirmed that tumor cells expressing the miR-424 inhibitor packaged the inhibitor into secreted EVs (miR-424i-EV) (Figure S3H). Further, we validated that miR-424i-EV and miR-424KO-EV (EVs derived from miR-424KO tumor cells) lack functional miR-424 and cannot regulate CD28 and CD80 (Figure S3I). In a human *in vitro* model, we incubated Jurkat T cells with the Raji Burkitt's lymphoma cell line to mimic a T cell-APC cell interaction. Addition of TEVs derived from the human CRC cell line HT29, which have normal miR-424, inhibited CD28 and CD80 expression on Jurkat and Raji cells, respectively (Figure 3G). Transfecting HT29 with a miR-424 knock-down construct abolished this effect, while transfection with a control did not (Figure 3G). Next, we tested the effect of TEVs generated from CRC patient derived tumor organoids on T-cell activation, which is indicated by IL-2 production. Tumor organoids retain the heterogeneity of tumors and are considered to be advanced *in vitro* models (Kopper et al., 2019; van de Wetering et al., 2015). In this experiment, we used a transwell assay with Jurkat/Raji cells in the upper chamber and CRC organoids in the lower chamber (Figure 3H). The presence of tumor organoids reduced IL-2 production from the Jurkat-Raji conjugation system. Noticeably, blocking EVs secretion by GW4869 rescued IL-2 production (Figure 3H).

Finally, we investigated how miR-424 expression is regulated in human CRC cells. Hypoxia is a key feature that differentiates tumors from normal tissues and is commonly observed in human

CRC(Baba et al., 2010). Induction of hypoxia stabilized the transcription factor HIF-1 α expression in HT29 cells and led to the upregulation of miR-424 and increased EV production (Figure S3J). Our findings align with previous reports in other experimental systems(Ghosh et al., 2010; King et al., 2012; Zhang et al., 2014). Collectively, these data reveal that hypoxia in tumor cells causes upregulation of miR-424 that is delivered to tumor infiltrating T cells and DCs by TEVs where it suppressed CD28-CD80/86 costimulatory signaling.

Blocking tumor cell derived miR-424 inhibits tumor development in an immune-dependent manner

Because TEVs with miR-424 suppressed the CD28-CD80/86 costimulatory pathway, we hypothesized that tumor-derived miR-424 impacts mouse CRC tumor development *in vivo*. Stably blocking the expression of Rab27a (a protein regulating exosome secretion) using shRAB27a reduced TEV production by MC38 tumor cells (Figure 4A). Suppressing TEVs' secretion significantly reduced MC38 tumor growth *in vivo* (Figure 4B). In another experiment, mice were subcutaneously injected with tumor cells that cannot produce endogenous miR-424 containing TEVs. The resultant tumors were then injected with TEVs containing functional miR-424 with a subsequent significant increase in tumor growth (Figure 4C). These data demonstrate that miR-424 in TEVs accelerates tumor development *in vivo*.

Next, we tested the impact of tumor cell derived miR-424 on tumor formation and growth. Notably, when functional miR-424 was depleted in tumor cells and TEVs, tumor formation and growth rates decreased significantly (Figures 4D and S4A). In the host mice that rejected miR-424i tumors (lacking functional miR-424), we observed an antitumor specific immune response (Figures 4E and S4B). In the TdLNs and spleens of these mice, we found an expansion of gp70 tumor antigen specific CD8⁺ T cells (Figure 4E). gp70 is a known tumor antigen present in CT26 cells. We achieved a similar result in the MC38 tumor model, this time p15E tumor antigen specific tetramer staining (Figure S4B). These data indicated that a robust antitumor immune response could contribute to the rejection of tumor formation in those mice. miRNAs have multiple autonomous targets in cell function. Therefore, to confirm that the antitumor immunity was the predominant reason for the slower development of tumors lacking functional miR-424, we repeated the experiments in severe combined immunodeficient (SCID) mice, *Cd28*^{-/-} mice, and *Cd80/86*^{-/-} mice (Figures 4F and S4A). Tumor growth rate, proliferation (Ki-67⁺), and apoptotic cell (cleaved-caspase-3⁺) frequency were similar among tumors with/without functional miR-424 in SCID mice (Figure 4F). Blocking miR-424 did not alter tumor growth in *Cd28*^{-/-} and *Cd80/86*^{-/-} mice (Figure S4A). Transcriptome analyses of tumor cells revealed that blocking functional miR-424 did not

extensively regulate cell-autonomous gene expression in tumor cells (Figures 4G and S4C-E). Taken together, these results indicated that blocking tumor cell-derived miR-424 effectively inhibits tumor development in immune competent mice by a CD28-CD80/86 dependent manner.

Blocking tumor cell derived miR-424 enhances adaptive antitumor immunity and sensitizes advanced tumors to ICBT

Since blocking tumor cell derived miR-424 suppressed tumor growth by an immune dependent mechanism, we then analyzed the impacts of tumor cell derived miR-424 on anti-tumor immune effectors. Immunohistological analyses on the CT26 and MC38 tumor sections showed that decreasing tumor cell-derived miR-424 led to a higher T-cell infiltration (Figures 5A and S5A). T cells were observed in both peri- and intra-tumor areas (Figures 5A and S5A). Analyses of antitumor immune landscapes were performed in tumor tissues, TdLNs, and the spleens of tumor-bearing mice (Figures 5B-C and S5B-D). Blocking tumor cell-derived miR-424 predominantly altered the antitumor immune landscape in both CT26 and MC38 tumor microenvironments (Figures 5B and S5B) but not in the TdLNs and spleens of tumor-bearing mice (Figures 5C and S5C-D). We found that T cells in tumors that lack miR-424 had elevated CD28 expression compared to T cells in control tumors. Similarly, the CD80 expression on DCs in miR-424 blocked tumors was higher than in control tumors (Figures 5B and S5B). A higher concentration of IL-2 was observed in miR-424 blocked tumors as well (Figures 5B and S5B). Other parameters such as total immune infiltration, T-cell infiltration and proliferation were enhanced by blocking tumor cell-derived miR-424 (Figures 5B and S5B). Remarkably, the major immunosuppressive mechanisms, such as tumor tissue PD-L1 expression and PD-1 expression on CD4⁺ T cells, were higher in the miR-424 blocked tumors (Figures 5B and S5B). The frequencies of T_{reg} and myeloid-derived suppressor cells (MDSCs) showed a trend of increase (Figures 5B and S5B). These mechanisms indicate a potential negative feedback response.

Our observations point out that the expression of CD28 on T cells and CD80 on DCs was downregulated in advanced tumors with elevated miR-424 (Figures 2G and Figure S2C). Because CD28 and CD80 are directly involved in the efficacy of anti-PD-1/PD-L1 treatments (Hui et al., 2017; Kamphorst et al., 2017; Sugiura et al., 2019; Zhao et al., 2019), we reasoned that blocking tumor cell-derived functional miR-424 would enhance tumor response to ICBT. We tested anti-PD-1/CTLA-4 treatments in early stage tumors and established tumors with/without functional miR-424 expression (Figures 5D and 5SE). Notably, anti-PD-1/CTLA-4 treatments in established WT tumors were less effective than in early stage tumors (Figures 5D and 5SE). In contrast, blocking tumor cell-derived miR-424 in combination with ICBT caused tumor rejection or reduction in established

tumor growth in WT mice. However, the treatment is ineffective in *Cd28*^{-/-} mice and *Cd80/86*^{-/-} mice even with blocked miR-424 in tumor cells (Figures 5D and 5SE). Systemic assessments by mass cytometry demonstrated distinctive antitumor immune landscapes in anti-PD-1/CTLA-4 treated established tumors with/without miR-424 (Figure 5E). A favorable phenotype with high tumor infiltrating T-cell frequency and function was only seen in treated tumors that lacked functional miR-424 (Figure 5E-F). These data support the significance of tumor cell-derived miR-424 in determining tumor response to ICBT.

Depleting functional miR-424 induces antitumor immunogenicity of TEVs

Wild type TEVs are generally considered immunosuppressive (Daassi et al., 2020; Whiteside, 2016). On the other hand, TEVs do contain tumor antigens that can induce an antitumor immune response (Diamond et al., 2018; Horrevorts et al., 2019; Poggio et al., 2019; Wolfers et al., 2001). Therefore, we speculated that depleting immunosuppressive factors, such as miR-424, would potentially enhance the immunogenicity of TEVs. To address this, we systemically administered TEVs with/without functional miR-424 to naïve mice. We first validated that the systemically administered TEVs could be taken in by immune cells in the peripheral lymphatic organs (Figure 6A). We then found that administration of TEVs lacking miR-424 (termed modified TEVs) could stimulate the expansion of tumor antigen specific CD8⁺ T cells in peripheral lymphatic organs of naïve mice without inducing extensive non-specific T-cell activation (Figure 6B). Moreover, to investigate whether a modified TEVs induced tumor specific immune response can reject tumors *in vivo*, we induced tumors in mice preconditioned with TEVs (Figure 6C). All mice treated with saline and TEVs with functional miR-424 developed CT26/MC38 tumors (Figure 6C). Remarkably, mice treated with modified TEVs were immunized to the tumor cells or only developed minimal CT26/MC38 tumors (Figure 6C). Repetition in *Tlr4*^{-/-} mice showed similar results, indicating that the antitumor specific immune response, rather than potential non-specific immune responses, accounts for the protective effects of modified TEVs (Figure 6C). In SCID mice, *Cd28*^{-/-} mice, and *Cd80/86*^{-/-} mice, the treatment failed to prevent tumor formation (Figure 6C), indicating that the effect is highly dependent on the CD28-CD80/86 costimulatory pathway.

We monitored body weight, blood thrombotic status, and serum cytokine levels of recipient mice (Figure S6A-C) to evaluate treatment safety. No differences were observed between untreated and treated groups (Figure S6A-C). Histological analysis of the major organs was performed and confirmed that the short-term modified TEV treatment (2 doses in one week, i.v., 10µg protein per dose) did not cause severe damage to the heart, liver, lung, kidney, small intestine, or spleen

(Figure S6D). Cumulatively, our findings highlighted that modified TEVs are immunogenic and can stimulate antitumor immunity in a safe manner.

Modified TEVs enhance ICBT efficacy in an advanced CRC pre-clinical tumor model

Given the potent antitumor immunogenic features of the modified TEVs, we further examined their therapeutic value in advanced CRC pre-clinical tumors. As a preliminary test, we administered the modified TEVs as a treatment for early (100-200mm³) and established (500-700mm³) subcutaneous tumors and found that the modified TEVs treatment alone was effective in minimal tumors, but not advanced tumors (Figure S7A). Since we have shown that the advanced tumors have high levels of immunosuppressive factors (Figure 2G), we reasoned that the antitumor immune response initiated by modified TEVs was not sustained in the advanced tumors. Thus, we proposed to combine the modified TEVs with ICBT to treat immunosuppressive tumors.

To mimic human late-stage CRC, we developed a cecum based orthotopic transplantation pre-clinical model (Cespedes et al., 2007; Song et al., 2018; Terracina et al., 2015). Both established primary tumor in cecum and metastatic lesions in the liver and peritoneal cavity were observed in this model (Figure S7B-C), indicating the advantages of using it to study treatment for stage IV CRC. For established tumors, two doses of modified TEVs were given during the 1st week of treatment for induction and anti-PD-1/CLTA-4 were given twice a week until the endpoint for sustention (Figure 7A). Notably, combination of modified TEVs with ICBT significantly reduced tumor metastatic burden and extended overall survival by more than 50% compared with ICBT only group (Figure 7B-C). Tumors in each group were subjected to immune cell analyses which showed that the combination treatment with modified TEVs and ICBT stimulated the most potent antitumor immunity with the highest T-cell infiltration frequency and activity (Figures 7D and S7D). Thus, combination therapy using modified TEVs that can stimulate tumor specific T-cell expansion in peripheral lymphatic organs (Figure 6B-C) along with ICBT that depletes immunosuppressive factors in the TME, highlights a potentially novel therapeutic approach to delay progression in late-stage CRC.

DISCUSSION

In this study, we demonstrate a critical immunosuppressive role of tumor cell-derived miR-424 as a negative regulator of CD28-CD80/86 costimulatory signaling in tumor infiltrating T cells and DCs. Blocking miR-424 in TEVs alleviated the immunosuppressive effects and enhanced the immune stimulatory effects of TEVs, making them a potential adjuvant treatment for ICBT used to treat

CRC. It was noteworthy that the CT26 and MC38 cells used in this study are representative of CRC-MSS and -MSI phenotypes, respectively(Efremova et al., 2018). Thus, our data revealed a shared mechanism of T-cell costimulation in these CRC subtypes.

In both men and women, CRC is the third most common type of cancer(Siegel et al., 2020). CRC patients were among the first cohort of cancer patients tested for ICBT; however, the majority of late stage treatment refractory CRC patients were unresponsive to ICBT in the early clinical trials. After several trials, it became evident that almost all responders had the MSI subtype(Brahmer et al., 2010; Le et al., 2015). Although the mutational loads in different CRC subtypes are considered one of the major determining factors of ICBT response(Zhao et al., 2018), other mechanisms regulating the immune response likely exist. Indeed, around 40-70% of the late stage MSI-CRC with high mutational load failed in ICBT(Le et al., 2015; Oliveira et al., 2019). Furthermore, MSS CRC that are considered resistant to ICBT can still have a high frequency of tumor infiltrating T cells(Kikuchi et al., 2019; Mlecnik et al., 2016; Pages et al., 2018). Our observation in TCGA CRC dataset and a cohort of 68 CRC patient samples validated that a proportion of the MSS tumors have comparable T-cell infiltration to MSI tumors (Figure 1). This evidence supports the hypothesis that independent of the MSI/MSS classification, there are other potential mechanisms controlling the adaptive immune response in CRC which need further investigation.

ICBT functions through blocking the inhibitory immune checkpoint molecules expressed on tumor cells, T cells, and other TME components. However, to rescue exhausted T cells by blocking PD-1/PD-L1 signaling, positive costimulation from the CD28-CD80/86 pathway is required(Kamphorst et al., 2017). Furthermore, biochemical studies demonstrated that T-cell expressed CD28 is a primary target for PD-1 mediated inhibition(Hui et al., 2017). In infectious conditions, CD28 expression is also required after T-cell priming for helper T-cell-mediated immune protection(Linterman et al., 2014) and the presence of CD28-mediated signaling prevents induction of anergy in T cells(Harding et al., 1992). All of these observations prompted us to investigate whether the CD28-CD80/86 costimulatory signaling is a prerequisite for ICBT response and whether this signaling is preserved in the CRC immune microenvironment. Remarkably, our analysis of human CRC indicated that some tumor infiltrating T cells and DCs lack expression of CD28 and CD80/86, respectively (Figure 1). Moreover, depleting or blocking the CD28-CD80/86 costimulatory signaling nullified the efficacy of ICBT in mouse CRC models that have immune cell infiltrations (Figure 1). Our findings are consistent with early reports in non-small cell lung cancer and melanoma (Kamphorst et al., 2017; Li et al., 2010) and support the hypothesis that the lack of intact CD28-CD80/86 costimulatory signals on infiltrated T cells and DCs restricts ICBT response in human CRC.

In non-cancerous conditions, CD28 expression levels decrease during antigenic exposure but rapidly revert back to pre-stimulation levels. However, sustained T-cell stimulation causes CD28 downregulation and eventual loss. CD28 downregulation with T-cell activation predominantly involves transcriptional repression and increased protein turnover as a T-cell intrinsic negative feedback mechanism(Weng et al., 2009). Biosynthesis and expression of CD80/86 on DCs is stimulated by antigen presentation and is associated with DC maturation. In cancerous conditions, the effects of tumor and stromal cells on T cells and DCs through direct contact and/or paracrine mechanisms cannot be ignored(Xie et al., 2019; Zhao and Subramanian, 2017, 2018). Our previous studies and other reports have established that abnormal expression of miRNAs is associated with CRC pathobiology(Li et al., 2014; Moridikia et al., 2018; Sarver et al., 2009). Therefore, we sought to investigate tumor cell-secreted miRNAs as a tumor intrinsic mechanism of CD28-CD80/86 downregulation in immune cells. We identified that miR-424s negatively regulate CD28 and CD80 expression (Figure 2). miR-424 was upregulated in CRC compared to normal colon, T cells, and DCs (Figures 2 and 3). In tumors, miR-424 was upregulated in tumor cells and was transported to surrounding T cells and DCs via TEVs, leading to downregulation of CD28 and CD80 proteins and defective T-cell function (Figure 3). Increased miR-424 levels in tumor cells were induced by hypoxia and accompanied by immune exclusion (Figure 2 and 3). These data validated that tumor intrinsic mechanisms can be an initiating factor for immunosuppression. Of course, we cannot exclude other unknown mechanisms that upregulate miR-424. The immune editing during tumor development may also contribute to the selection and accumulation of miR-424 producing tumor cells.

In mouse tumors, depleting or blocking miR-424 in tumor cells significantly delayed tumor growth in an immune-dependent manner (Figure 4). Immune landscape analyses demonstrated that lack of tumor derived miR-424 rescued CD28 expression on both tumor infiltrating CD4⁺ and CD8⁺ T cells. CD80 expression on the tumor infiltrating CD103⁺ DCs that are critical for tumor antigen presentation and effector T-cell trafficking(de Mingo Pulido et al., 2018; Roberts et al., 2016; Salmon et al., 2016; Spranger et al., 2017) were increased in tumors lacking miR-424. Notably, T-cell frequency and function were significantly higher in those tumors. As a consequence, lack of tumor-derived miR-424 and restoration of CD28-CD80/86 signaling reversed tumor intrinsic resistance to anti-PD-1/CTLA-4 treatment (Figure 5). Our data reinforces previous findings that intact CD28-CD80/86 costimulatory pathways could attenuate PD-1 mediated T-cell exhaustion and were associated with better response to anti-PD-1 treatment(Fenwick et al., 2019; Kamphorst et al., 2017; Mizuno et al., 2019). Recent mechanistic studies describing the immunostimulatory and therapeutic significances of CD80 expression on APCs(Sugiura et al., 2019; Zhao et al., 2019)

also support our findings. In summary, our studies establish that TEV-delivered miR-424 is a crucial mechanism causing defective CD28-CD80/86 costimulatory signaling thus inducing ICBT resistance in human CRC.

Blocking tumor cell derived miR-424 restored the defective CD28-CD80/86 costimulatory signaling in tumors and resulted in a better response to ICBT (Figure 5). However, in clinical conditions, it is difficult to genetically alter tumor cells or specifically block miRNAs in the TME. TEVs carry tumor antigens and are being tested as potential cell-free cancer vaccines (Diamond et al., 2018; Wolfers et al., 2001). Therefore, to translate our mechanistic findings to potential therapeutic approaches, we considered enhancing the immunostimulatory aspects of TEVs by eliminating the miR-424 induced immunosuppression. Our modified TEVs (without immune suppressive miR-424) stimulated a tumor-specific immune response that was independent of the tumor's endogenous antigen presentation and T-cell priming. Systemic administration of modified TEVs in naïve mice induced tumor-specific immune responses and are protective upon tumor cell challenge in the preconditioned mice (Figure 6). Careful monitoring of the health of the recipient mice indicated there were no serious adverse side effects of this treatment (Figure S6). A recent phase 2 stage clinical trial suggested that utilizing the anti-PD-1/CTLA-4 combination treatment as a neoadjuvant therapy in early stage CRC might have a better response than being used as an adjuvant therapy in late stage tumors (Chalabi et al., 2020). Therefore, we established an orthotopic CRC model that mimics human metastatic CRC tumors to test the therapeutic efficacy of modified TEVs. In such an aggressively progressing CRC model, combination of modified TEVs and ICBT resulted in an increased immune response and improved mouse survival rate over the treatment with ICBT alone (Figure 7). This is most likely due to the modified TEVs ability to stimulate anti-tumor specific T-cell responses which could then be sustained by ICBT. Similarly, a recent study using TEVs produced by irradiated mouse breast cancer cells demonstrated the enhanced costimulatory functions of DCs (Diamond et al., 2018). These findings together with our results underscore the potential of using modified TEVs with enhanced immunostimulatory effects in stimulating antitumor immunity and treating ICBT resistant tumors.

Our results have several important implications for clinical translation. First, we show that tumor cells may induce positive costimulatory pathway deficiency in tumor infiltrating immune cells. To revitalize the tumor infiltrating T cells, protecting or restoring the positive costimulatory pathways may be equally important as suppressing the inhibitory pathways. This concept is backed by a recent report using CD28 stimulatory mechanisms to synergize with anti-PD-1 blocking antibody (Fenwick et al., 2019). Second, our data showing that modified TEVs could stimulate anti-tumor T-cell responses and reverse ICBT resistance in advanced CRC models suggest potential

therapeutics for ICBT resistant tumors. The WT TEVs are considered as immunosuppressive in general(Iero et al., 2008; Kurywchak et al., 2018). However, recent findings identified the immunosuppressive factors in those TEVs and showed that blocking them can alleviate the immunosuppression(Chen et al., 2018; Kim et al., 2019; Poggio et al., 2019). Further, adding immunostimulatory factors into the TEVs can even enhance their ability to initiate immune responses(Diamond et al., 2018). Traditional TEV-related therapies used WT TEVs to pulse DCs *in vitro* and TEVs loaded DCs were infused to treat tumors(Bu et al., 2011; Gu et al., 2015; Zuo et al., 2020). This method avoids the potential negative effects of TEVs on the immune system *in vivo*. However, this approach is time consuming and did not consider the negative effects of WT TEVs that may impart functional defects in DCs, leading to treatment failure. Administration of the modified TEVs can minimize the negative effects of WT TEVs on immune response and, at the same time, efficiently stimulate tumor-specific immunity *in vivo*. Modified TEVs could be an alternative to the current TEVs based cancer treatments.

Taken together, our study reinforces previous reports regarding a positive costimulatory pathway and TEVs in ICBT(Kamphorst et al., 2017; Poggio et al., 2019). We deciphered a novel mechanism of CRC immune evasion and an innovative concept of modified TEV-based treatment. It is noteworthy that miR-424 may have strong effects on other immune cell populations or immune processes considering it has a large number of predicted immune gene targets. Our current results do not exclude the contribution of other potential immune evasive mechanisms of ICBT failure(Kalbasi and Ribas, 2020). A comprehensive understanding of immune suppressive mechanisms and accurate blocking of these suppressive factors is critical and necessary for a successful treatment. Finally, an array of other immunosuppressive factors have been identified in TEVs as well(Kim et al., 2019; Poggio et al., 2019; Ricklefs et al., 2018; Vignard et al., 2020). Blocking a bulk of these negative regulators and adding immunostimulatory factors may further enhance the efficacy of modified TEVs on stimulating anti-tumor immunity but more investigation is required.

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AUTHOR CONTRIBUTIONS

X.Z. and S.S. conceived and designed the experiments. X.Z. and D.W. performed all experiments

and data analyses. C.Y., and D.W. provided assistance in gene expression analysis and tumor organoid culture. X.Z., C.Y., D.W. and S.S. commented on the data. X.Z. and S.S. co-wrote the paper. S.S. supervised this project.

DECLARATION OF INTERESTS

The authors declare no competing interests

DATA AVAILABILITY

RNA sequencing data is deposited in SRA BioProject, accession number: PRJNA627790.

FIGURE LEGENDS

Figure 1. Expression of costimulatory molecules CD28, CD80, and CD86 on tumor infiltrating immune cells in human CRC

(A) Multiplex immunofluorescence staining of tumor infiltrating T cells (CD3, CD8) and myeloid cells (CD11b) in human CRC tissue samples. (23 MSI cases, 45 MSS cases, Mean±SEM, t-test, **** $p<0.0001$)

(B) Immune checkpoints expression profile in TCGA CRC dataset.

(C) Flow cytometry analysis of CD28, CD80 and CD86 on immune cells in fresh human CRC tissues. (Mean±SEM, n=10, t-test, * $p<0.05$)

(D) Multiplex immunofluorescence staining of CD28 on T cells and CD80/86 on DCs in a human CRC cohort (23 MSI cases, 45 MSS cases). Only T cells and DCs in the tumor center were scored. Magnification 400X.

(E) Impact of CD28 and CD80/86 expression on ICBT (anti-CTLA-4 + anti-PD-1) in subcutaneous tumors. ICBT restricted early stage tumor development in WT mice. However, *Cd28*^{-/-} mice and *Cd80/86*^{-/-} mice were resistant to ICBT. Antibody blocking of CD80 and CD86 induced tumor resistant to ICBT. (Individual data for each case, n=5, t-test, ** $p<0.01$, **** $p<0.0001$)

See also Figure S1 and Table S1.

Figure 2. miR-424 is highly expressed in human CRC and negatively regulates CD28 and CD80 expression

(A) Schematic of process to identify miR-424 as a mechanism to downregulate the CD28 costimulatory pathway in human CRC.

(B) miR-424 expression in human CRC and normal colon cases (TCGA dataset). (Kruskal-Wallis test, **** $p<0.0001$)

(C) In situ hybridization (ISH) of miR-424 expression in human tissues. Representative staining of normal colon (left panel) and CRC tissue (right panel). (n=10)

(D) qRT-PCR analysis of miR-424 expression in paired human CRC and normal colon tissues. Scale shows Fold change (FC).

(E) Dual luciferase reporter assay of miR-424 on the 3'UTR of human CD28 and CD80.

(Mean±SEM, n=3, t-test, ** $p<0.01$, **** $p<0.0001$, n.s. no significance)

(F) Primary human CD4⁺ and CD8⁺ T cells, and DCs transfected with miR-424 mimic showed lower CD28 and CD80 protein expression respectively. (Mean±SEM, n=3, t-test, * $p<0.05$, ** $p<0.01$)

(G) Heatmap representation of miR-424 expression level and the immune landscapes during various phases of CT26 tumor growth. (n=3, t-test, p -values listed)

See also Figure S2.

Figure 3. Tumor cell derived EVs transfer miR-424 to immune cells and downregulate CD28 and CD80

(A) Relative levels of miR-424 expression in human and mouse CRC cell lines, primary human and mouse T cells and DCs, and primary human CRC organoids.

(B) Representative photomicrograph of primary human CRC organoids.

(C) Confocal microscopy confirmed EV up take by T cells and DCs. CT26 cells derived EVs were stained with Dio dye and incubated with mouse naïve T cells and DCs.

(D) CT26 cells cocultured with naïve T cells and DCs in a transwell system with and without EV inhibitor GW4869. miR-424 levels in T cells and DCs in each experimental condition. (Mean±SEM, n=3, t-test, * $p<0.05$, ** $p<0.01$)

(E) Flow cytometry analysis showed GFP signal in a proportion of tumor infiltrating T cells and DCs of the CT26-CD63-GFP tumors. Wild type (upper row) and CD63-GFP transfected CT26 cells (lower row) were used to establish subcutaneous tumors. T cells and DCs in the CT26-CD63-GFP tumor draining lymph nodes (TdLNs) showed very weak GFP signal. (n=3 in each condition)

(F) miR-424 levels in T cells and DCs isolated from the spleen of naïve mice and subcutaneous tumors (established by wild type (WT) tumor cells or miR-424 knockout (KO) tumor cells) by flow sorting. (Mean±SEM, n=3, t-test, * $p<0.05$)

(G) Expression levels of CD28 on Jurkat cells and CD80 on Raji cells in conjugation system: WT-EVs, miRi-Ctrl-EV, and miR-424i-EV derived from human HT29 CRC cells were used for each set. (Mean±SEM, n=3, t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

(H) Human CRC derived organoids were cocultured with the Jurkat and Raji cells conjugation system in a transwell system with or without GW4869. IL-2 production in the coculture system was measured. (Mean±SEM, n=3, t-test, * $p<0.05$)

See also Figure S3.

Figure 4. Tumor EVs with functional miR-424 accelerated tumor growth by suppressing antitumor immunity

(A) Knockdown of Rab27a significantly reduced EV production in MC38 cells. (Mean±SEM, n=3, t-test, * $p<0.05$)

(B) Growth curve of MC38 subcutaneous tumors established by MC38-Rab27aKD and MC38-Scramble-Ctrl cells. (Mean±SEM, n=5, t-test, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

(C) CT26 EVs with functional miR-424 (CRISPR-Ctrl EV) or without miR-424 (miR-424KO EV) were injected into the CT26-miR-424KO tumors in mice. Exogenous compensation of TEVs with

miR-424 enhanced growth of tumors without endogenous miR-424. (Mean±SEM, n=5, t-test, *** $p<0.001$, **** $p<0.0001$)

(D) CT26 cells with functional miR-424 (WT, miRi-Ctrl, CRISPR-Ctrl, miR-424OE) and CT26 tumor cells without functional miR-424 (miR-4242i, miR-424KO) were used to induce tumor formation in immune competent BALB/c mice. (n=10-20, Kaplan-Meier method for survival curve, log-rank test for survival analysis, **** $p<0.0001$)

(E) Antitumor specific immune response was detected in tumor free mice that rejected the miR-424i CT26 cells. (Mean±SEM, n=6, t-test, * $p<0.05$, *** $p<0.001$)

(F) Depletion of the functional miR-424 did not affect CT26 tumor growth pattern in SCID mice. Proliferation (ki-67) and apoptosis (cleaved-caspase-3) of tumor cells were not influenced by functional miR-424 depletion. (Mean±SEM, n=4, t-test, n.s. no significance)

(G) Upper panels: Differentially expressed genes in CT26 cells with and without functional miR-424. Lower panel: Gene Ontology analysis of biological pathways.

See also Figure S4 and Table S2-3.

Figure 5. Depletion of functional miR-424 enhanced the costimulatory molecules CD28 and CD80 expression, antitumor immunity, and response to ICBT.

(A) The overall T-cell infiltration in CT26 tumors with (WT, miRi-Ctrl) and without (miR-4242i) functional miR-424. T cells were observed in both peripheral and central region of tumors. (Mean±SEM, n=6, t-test, ** $p<0.01$ n.s. no significance)

(B) Heatmap representation of antitumor immune profiles in CT26 tumors without functional miR-424 and control tumors. Right panels: Levels of CD28 and CD80 expression on tumor infiltrating CD4⁺ and CD8⁺ T cells and CD103⁺ DCs. Intratumoral IL-2 concentration were measured in all three groups. (Mean±SEM, n=6, t-test, * $p<0.05$, ** $p<0.01$ n.s. no significance)

(C) Expression of CD28 on T cells and CD80 on antigen presenting cells (APCs) in tumor draining lymph nodes (TdLNs) and spleen (SPL) of MC38 tumor bearing mice. (Mean±SEM, n=6, t-test, n.s. no significance)

(D) In MC38 tumor model, ICBT is effective for treating the early stage tumors (volume ~100mm³) but not for controlling the large tumors (volume 500-700mm³). Blocking functional miR-424 in tumor cells enhanced the response of established tumors to ICBT in WT C57BL/6 mice, but not in *Cd28*^{-/-} mice and *Cd80/86*^{-/-} mice.

(E) Mass cytometry analysis of immune landscape in established tumors treated with ICBT. (n= 4 cases/condition). A representative t-SNE plots showing immune cell infiltration for each condition.

(F) Tumor infiltrating T cells proliferation induced by ICBT in established tumors without functional miR-424. (Mean±SEM, n=4, t-test, * $p<0.05$)

See also Figure S5.

Figure 6. TEVs without functional miR-424 are efficient in stimulating antitumor immune response

(A) Representative flow cytometry dot plots showing intravenously injected TEVs (CD63-GFP labeled) were taken by T cells and DCs in the peripheral lymphatic organs (n=3/group).

(B) Tail vein injection of TEVs (10µg) with different status of miR-424 function in naïve mice. TEVs without functional miR-424 (miR-424i-EV) stimulate tumor antigen specific (gp70) T-cell expansion in the peripheral lymphatic organs. Non-specific T-cell activation (CD62L⁺ frequency) was also evaluated. (Mean±SEM, n=4, t-test, * $p<0.05$ n.s. no significance)

(C) TEVs (10µg) with different miR-424 functional status were injected to naïve mice intravenously. Two weeks after injection, the mice were challenged with syngeneic tumor cells subcutaneously. Naïve WT mice preconditioned by TEVs without functional miR-424 (miR-424i-EV and miR-424KO-EV) showed lower tumor burden than mice preconditioned by TEVs with functional miR-424 or saline. The experiments were repeated in *Tlr4*^{-/-} mice, SCID mice, *Cd28*^{-/-} mice, and *Cd80/86*^{-/-} mice. The tumor burden was measured two weeks after tumor cells inoculation. Left half: CT26 model; Right half: MC38 model. (Mean±SEM, n=4-12, t-test, * $p<0.05$, *** $p<0.001$, **** $p<0.0001$, n.s. no significance)

See also Figure S6.

Figure 7. TEVs without miR-424 enhanced the efficacy of ICBT in established orthotopic tumors

(A) The schematic of treatment design. CT26-Luc cells were implanted to the cecum of naïve mice to establish an advanced stage orthotopic tumors. Tumor development was monitored by periodic *in vivo* imaging. Mice with established tumors were assigned to different treatments (see methods for dose details, representative images from each treatment groups are shown). Mice were either sacrificed after two weeks of treatment for evaluating disease burden and immune infiltration or followed up for survival analysis.

(B) Metastatic tumor burden evaluated after two weeks of treatment. (Mean±SEM, n=5, t-test for two group comparisons, one-way ANOVA for multiple groups comparison, * $p<0.05$, n.s. no significance)

(C) Kaplan-Meier overall survival analysis of mice in each group. The mean survival time listed in the table. (n=12, log-rank test for survival analysis, ** $p < 0.01$, n.s. no significance)

(D) Heatmap representation of immune profiles of tumors treated with different therapies. Right panels show quantitative analysis. (Mean $\pm$ SEM, n=5, t-test for indicated two groups, ** $p < 0.01$, *** $p < 0.001$, n.s. no significance)

See also Figure S7.

Reference

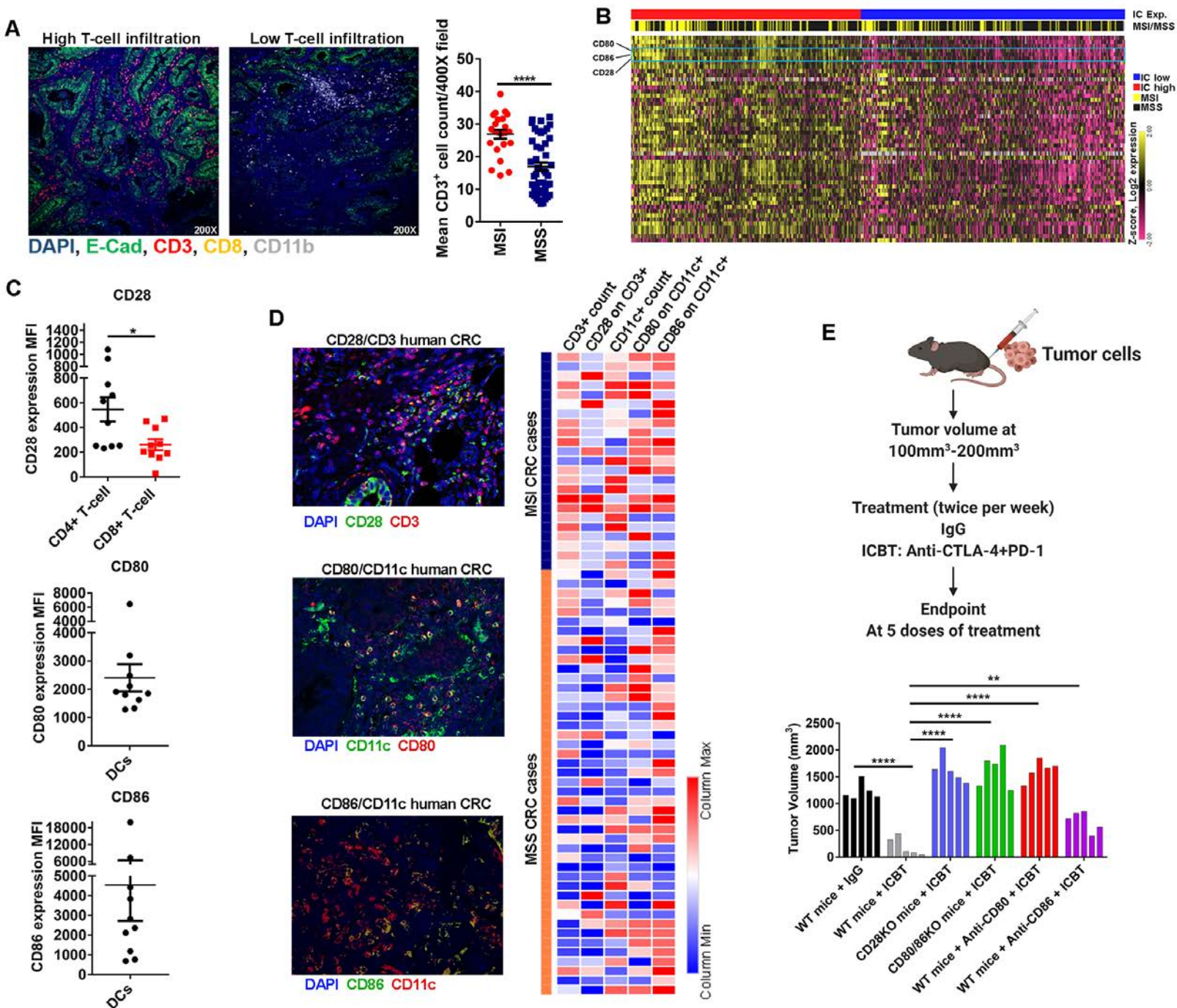
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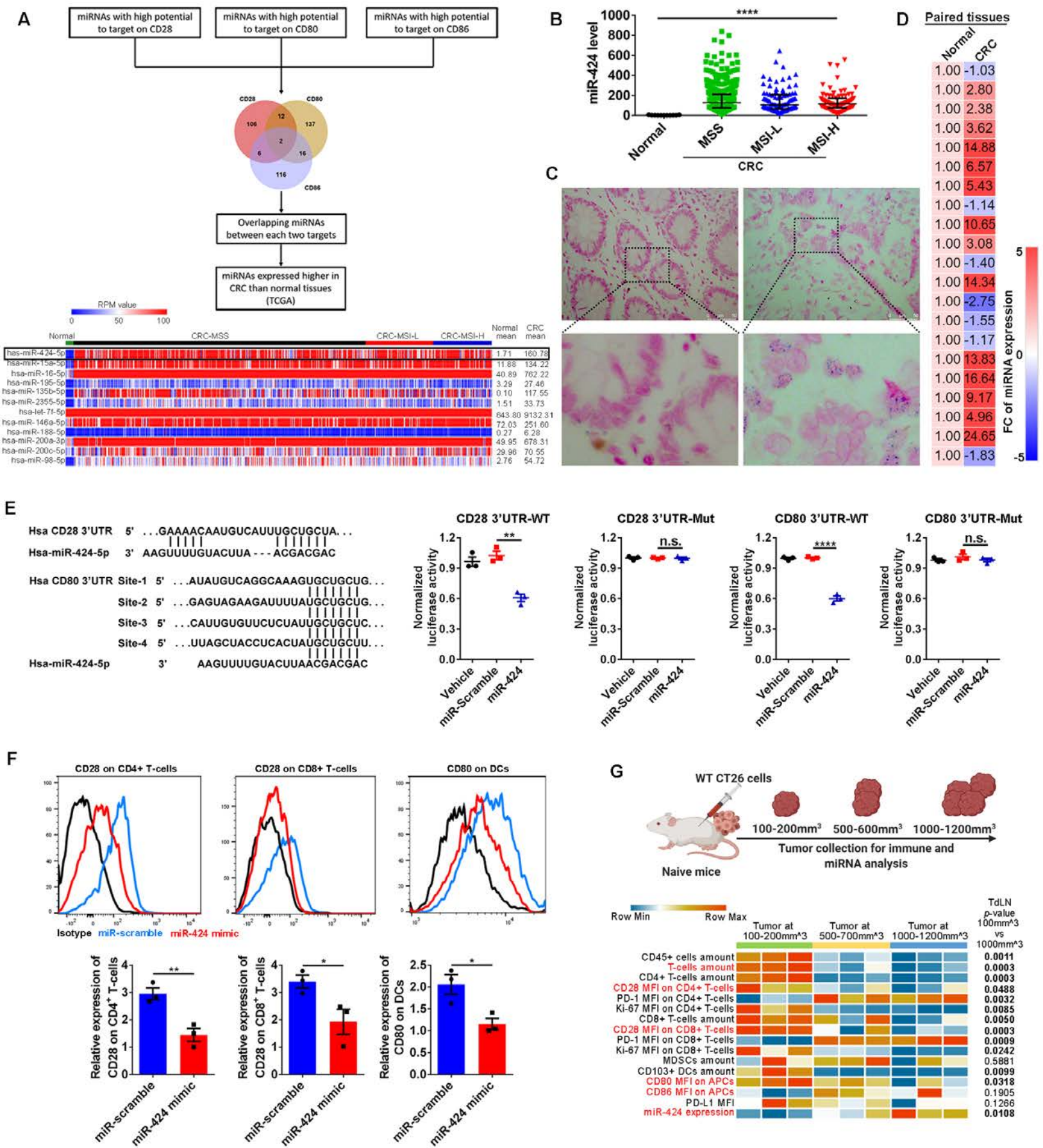
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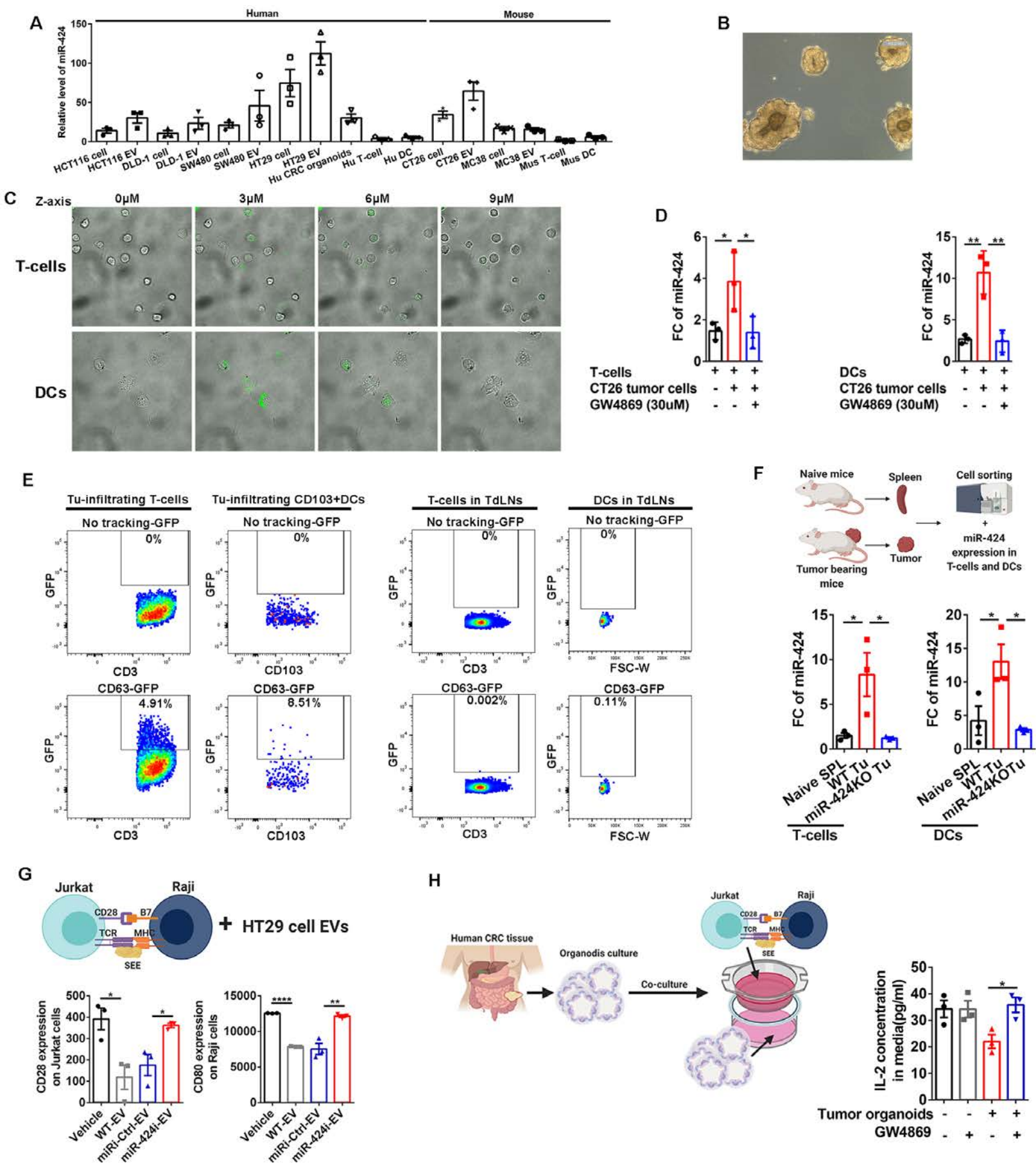
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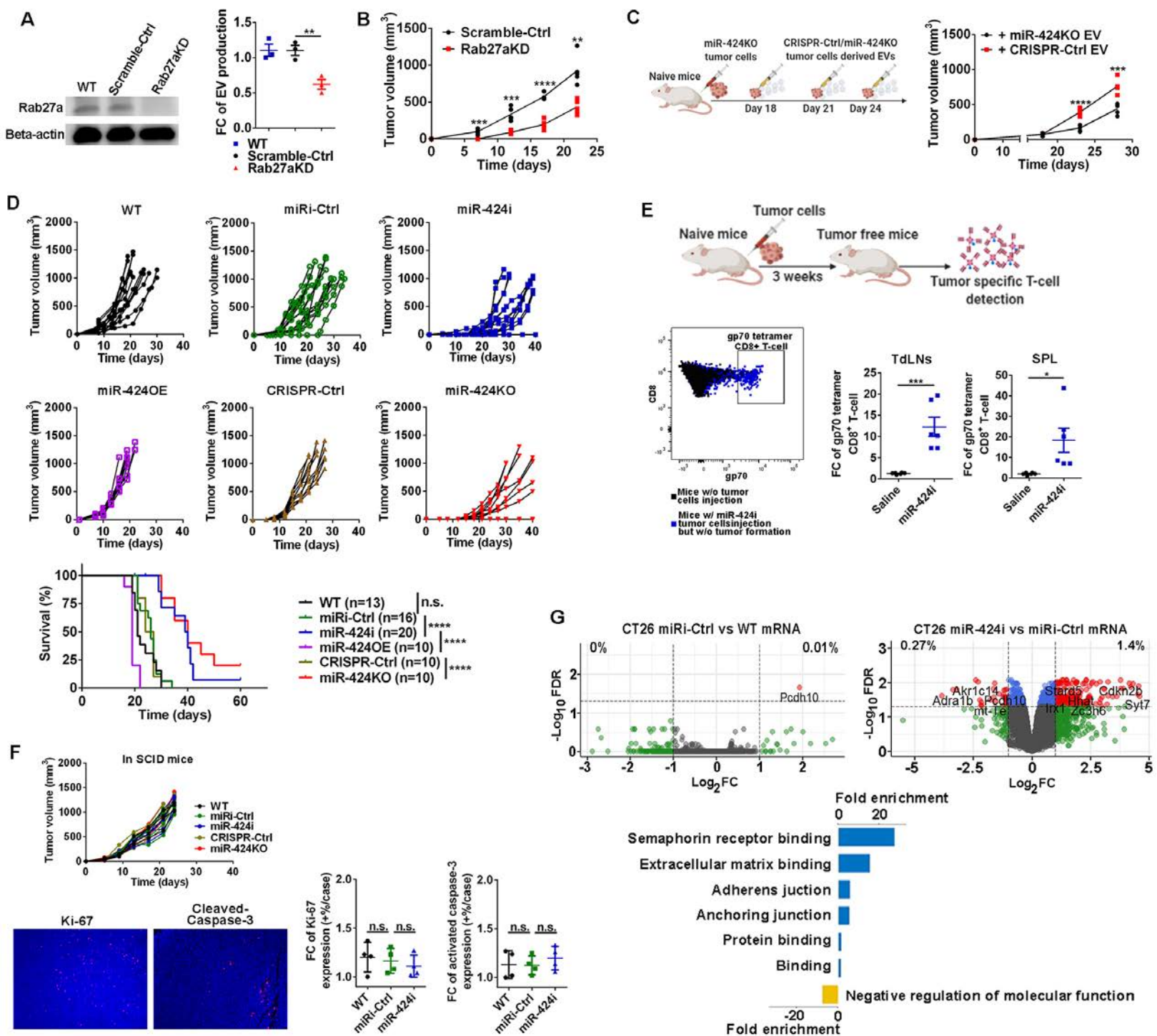
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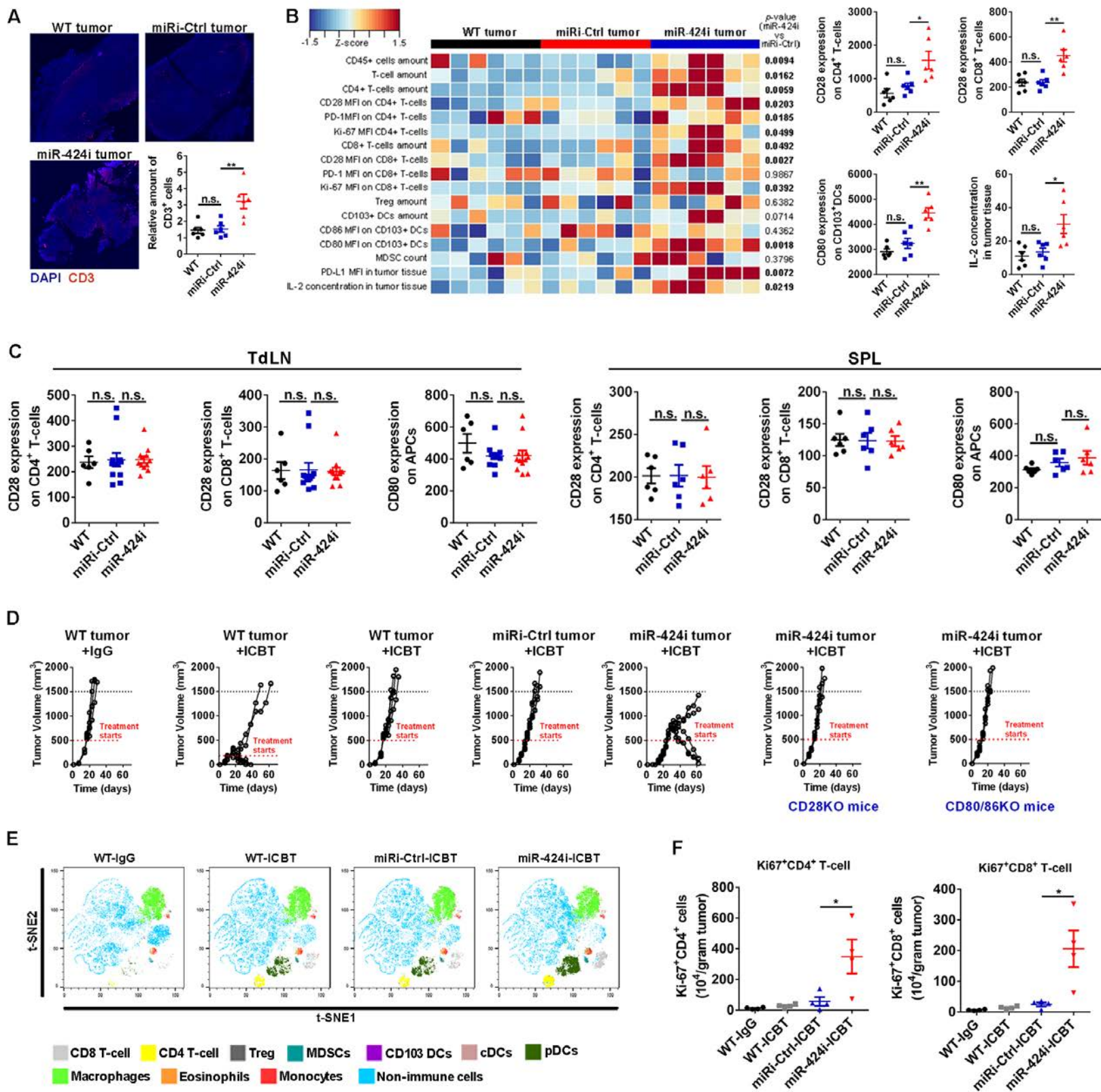
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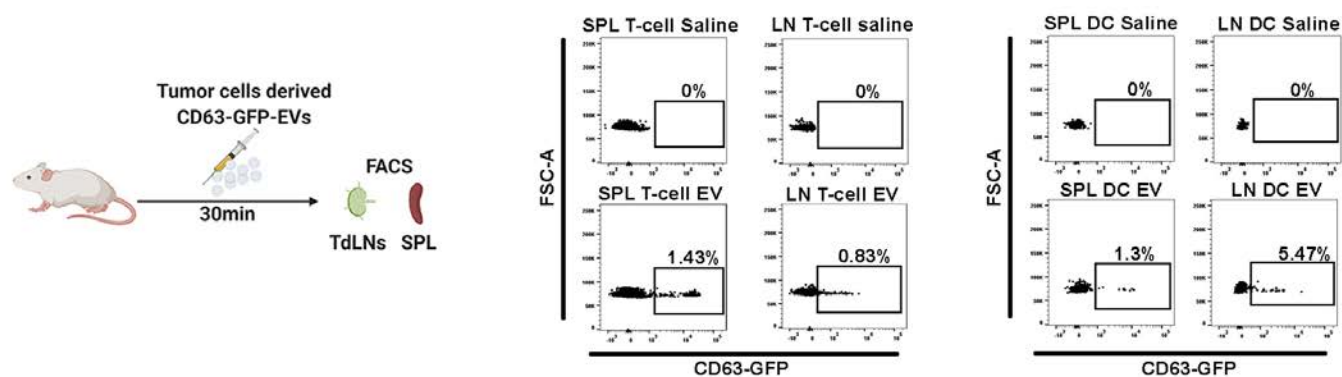




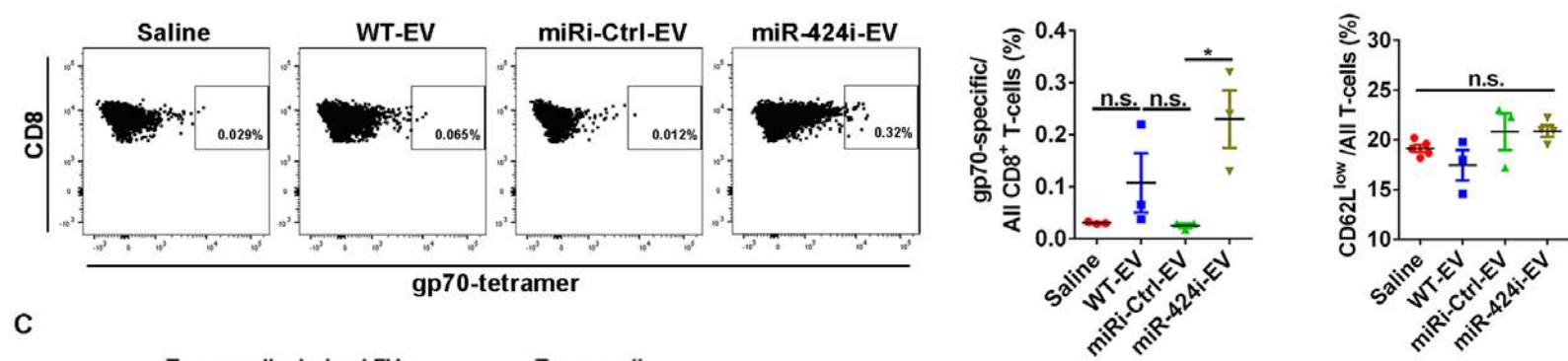




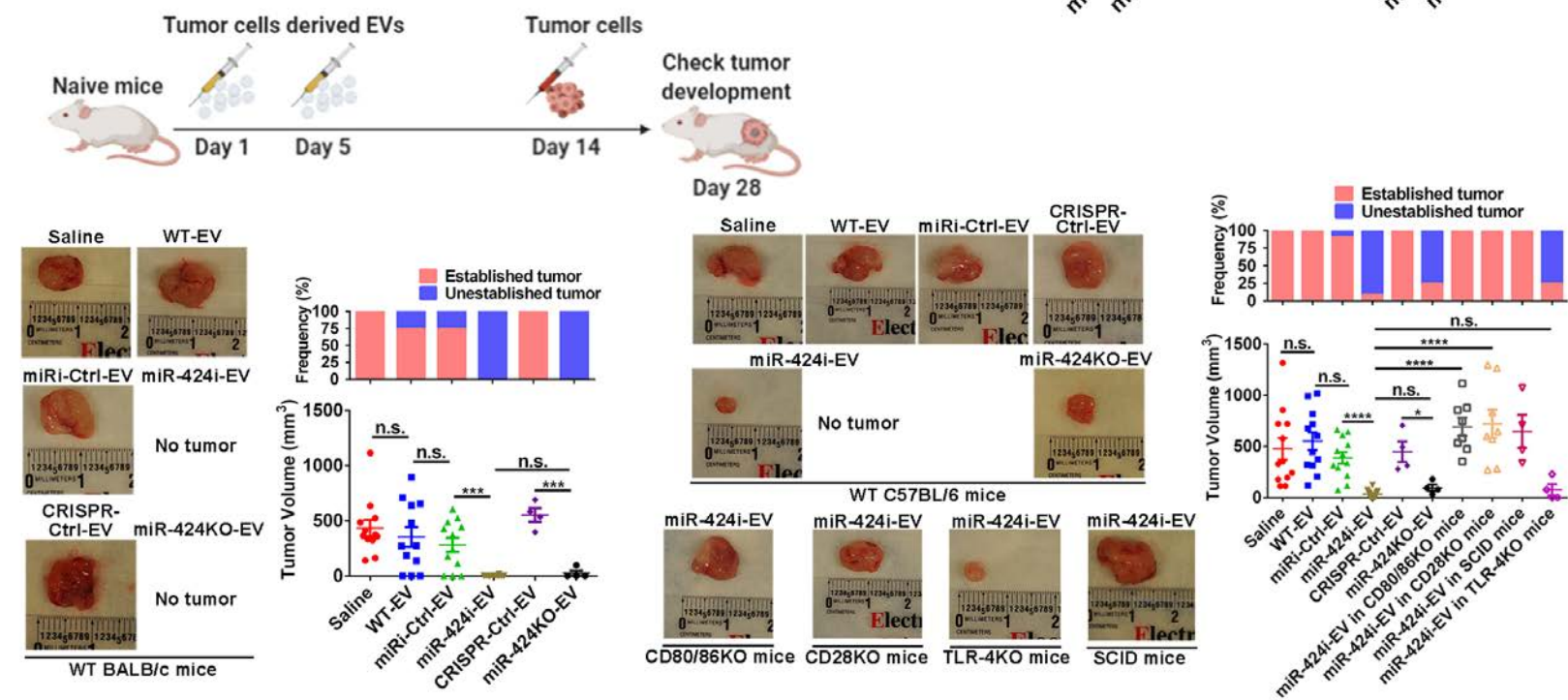
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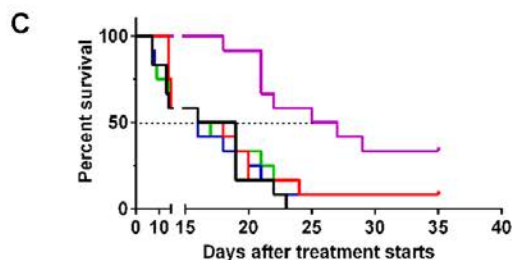
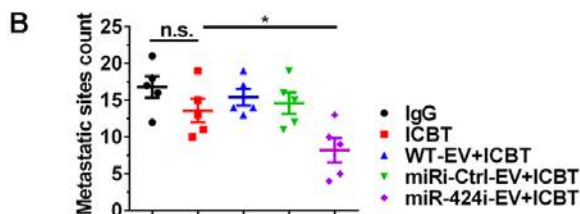
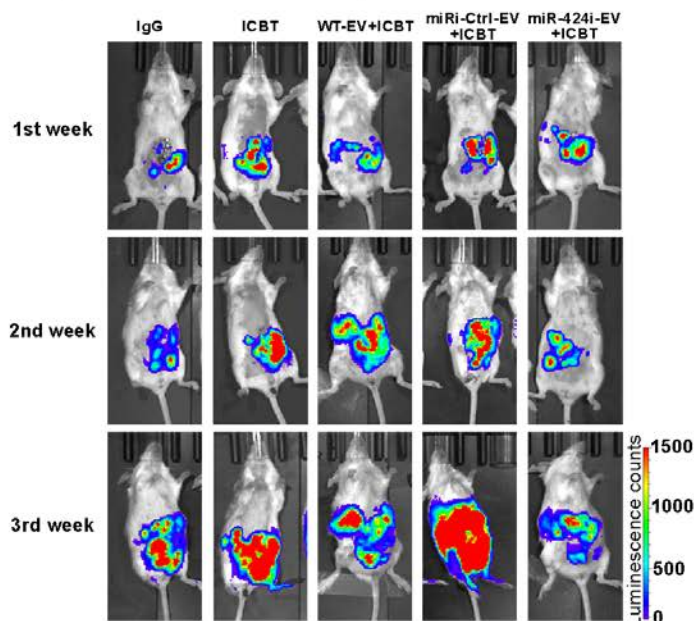
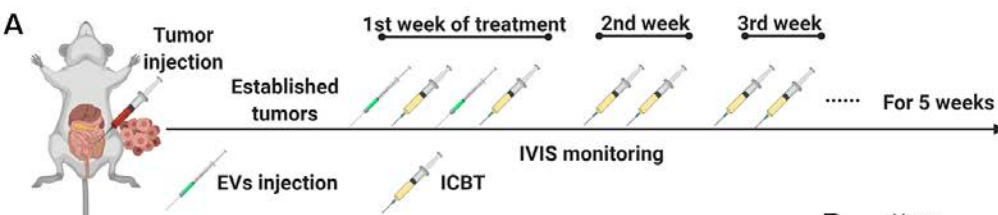


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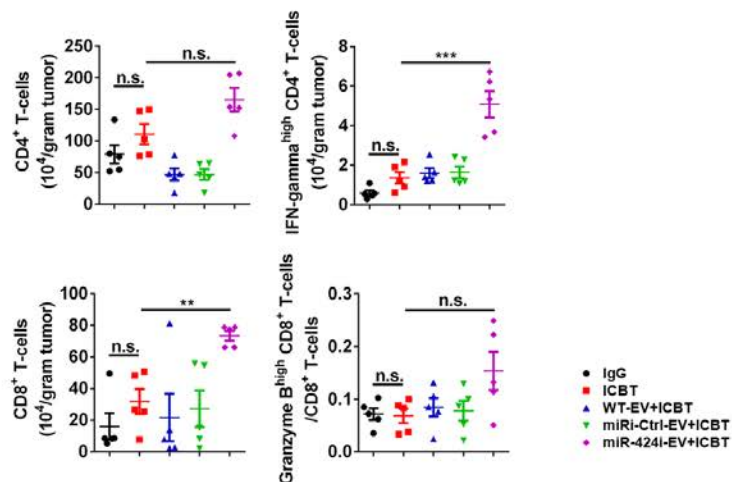
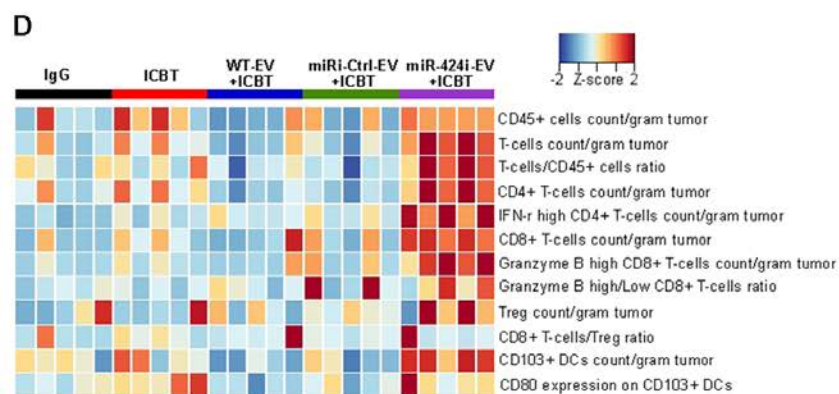


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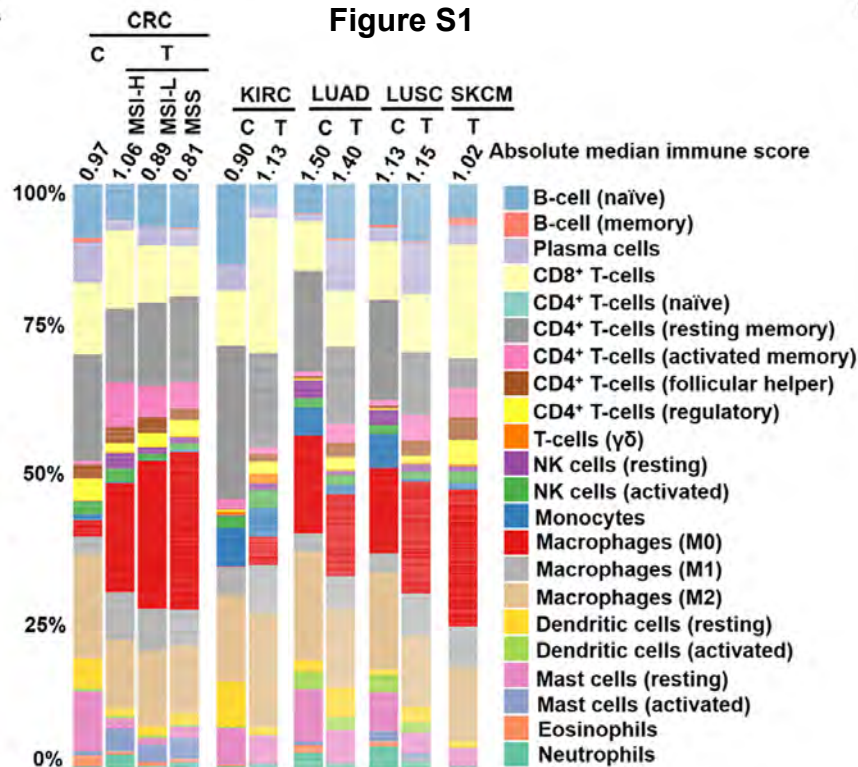


Treatment	Mean survival (days)
IgG (n=12)	15.9
ICBT (n=12)	18.7
WT-EV+ICBT (n=12)	17.2
miRi-Ctrl-EV+ICBT (n=12)	17.2
miR-424i-EV+ICBT (n=12)	27

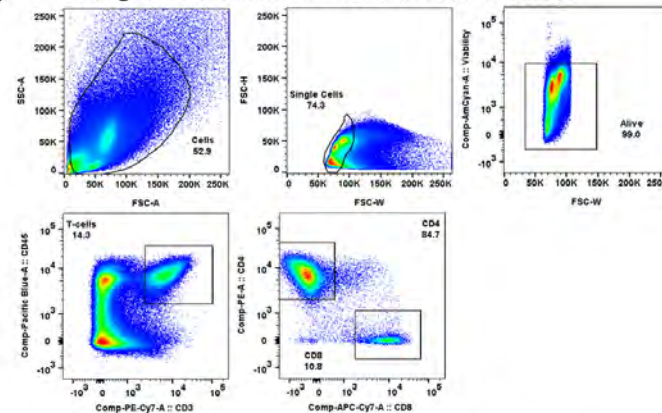


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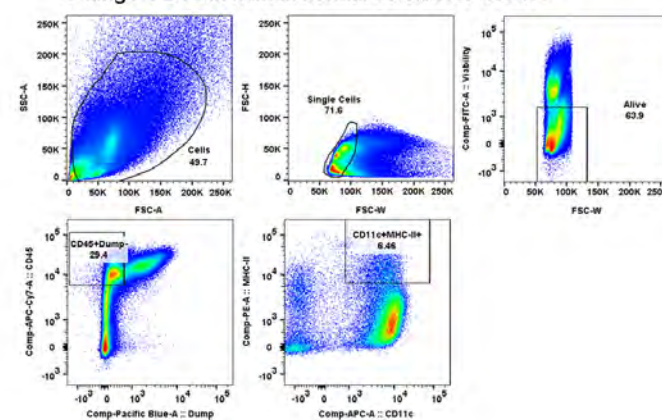
Figure S1



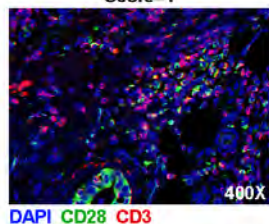
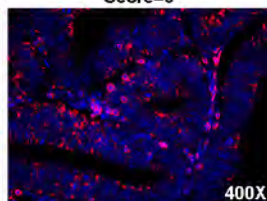
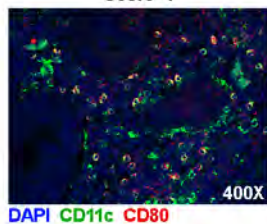
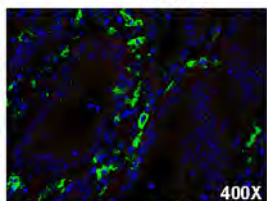
B Gating for T-cells in human normal colon/CRC tissues



Gating for DCs in human normal colon/CRC tissues

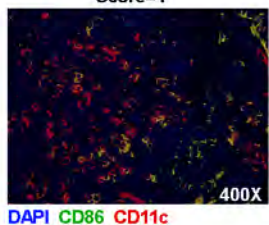
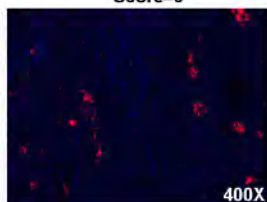


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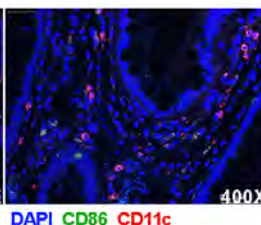
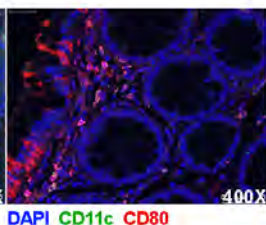
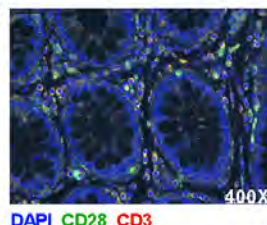
Field 1, CD28⁺CD3⁺/CD3⁺>5%
Score=1Field 2, CD28⁺CD3⁺/CD3⁺<5%
Score=0Field 1, CD80⁺CD11c⁺/CD11c⁺>20%
Score=1Field 2, CD80⁺CD11c⁺/CD11c⁺<20%
Score=0

For all markers:

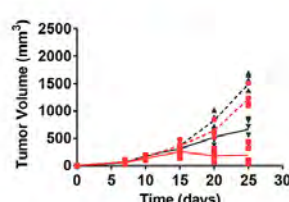
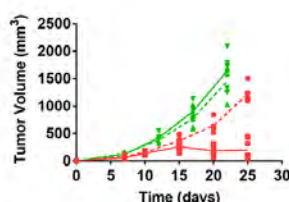
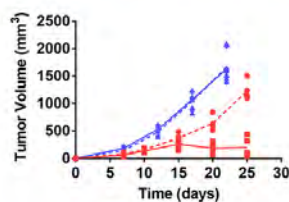
Score of the case = Sum of 5 fields / 5

Field 1, CD86⁺CD11c⁺/CD11c⁺>20%
Score=1Field 2, CD86⁺CD11c⁺/CD11c⁺<20%
Score=0

D



E



F

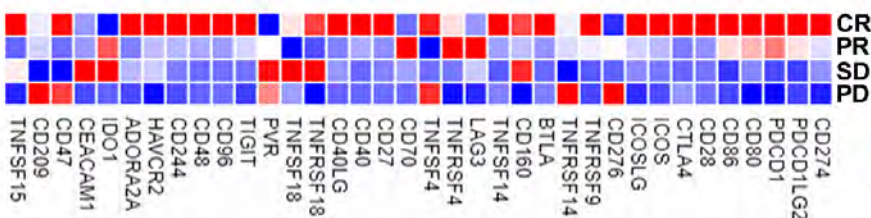


Figure S1. Expression of costimulatory molecules CD28, CD80, and CD86 on tumor infiltrating immune cells in human CRC. Related to Figure 1.

(A) The total immune infiltration and proportion of immune cells were estimated in CRC, kidney renal clear cell carcinoma (KIRC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and skin cutaneous melanoma (SKCM), through TCGA database analysis.

(B) Representative gating strategy used to identify T-cells and dendritic cells (DCs) in human tissues.

(C) Representative photos showed the scoring strategy to measure the expression of CD28 on CD3⁺ cells, and CD80 and CD86 on CD11c⁺ cells. The final score of each case is calculated as the mean score of five independent 400X field.

(D) Representative staining of CD28 on CD3⁺ cells, and CD80 and CD86 on CD11c⁺ cells in human normal colon tissues.

(E) The individual tumor growth curves of figure 1E.

(F) Co-stimulatory molecules expression profiling was analyzed in non-small cell lung cancer, head and neck squamous cell cancer, and melanoma patients after anti-PD-1 treatment. The heatmap showed a higher trend of CD28 and CD80/86 expression in patients with complete response (CR), partial response (PR), and stable disease (SD) than in patients with progressive disease (PD).

Figure S2

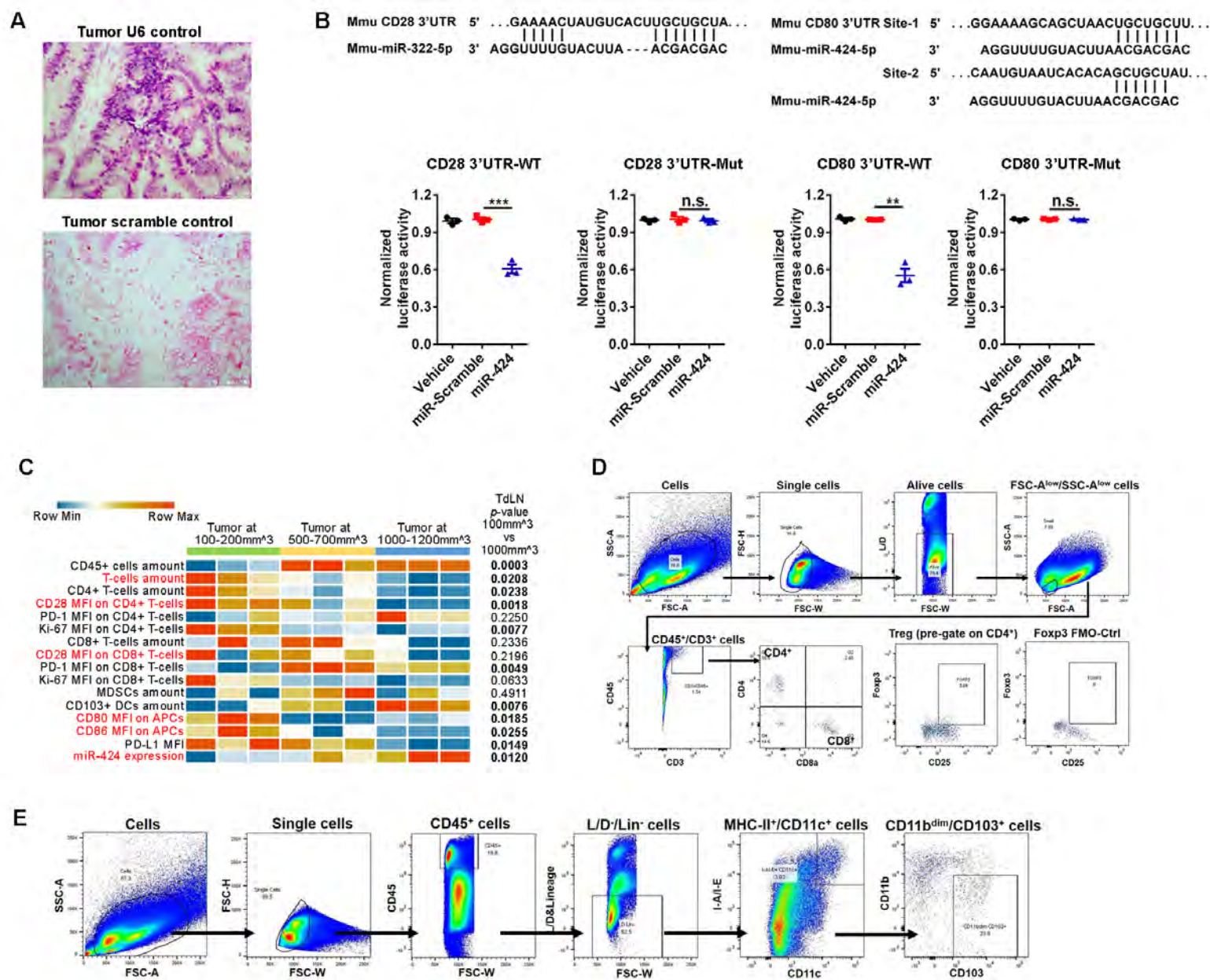


Figure S2. miR-424 is highly expressed in human CRC and negatively regulate CD28 and CD80 expression. Related to Figure 2.

- (A) The positive (U6) and negative (scramble) controls used for the ISH experiments.
- (B) Dual luciferase assay confirmed the binding of miR-424 on the 3'UTR of mouse CD28 and CD80. (Mean±SEM, n=3, t-test, ** $p<0.01$, *** $p<0.001$, n.s. no significance)
- (C) miR-424 expression level and the immune landscapes during the MC38 tumor progression, from early stage tumors to advanced stage tumors, were shown in the heatmap. (n=3, t-test, p -value was listed)
- (D) Representative gating strategy used to identify tumor infiltrating T-cells (FSC-A^{low}, SSC-A^{low}, CD45⁺, CD3⁺, CD4⁺/CD8⁺) in mouse tumor tissues.
- (E) Representative gating strategy used to identify tumor infiltrating CD103⁺ DCs (CD45⁺, MHCII⁺, CD11c⁺, CD11b^{dim}, CD103⁺) in mouse tumor tissues.

Figure S3

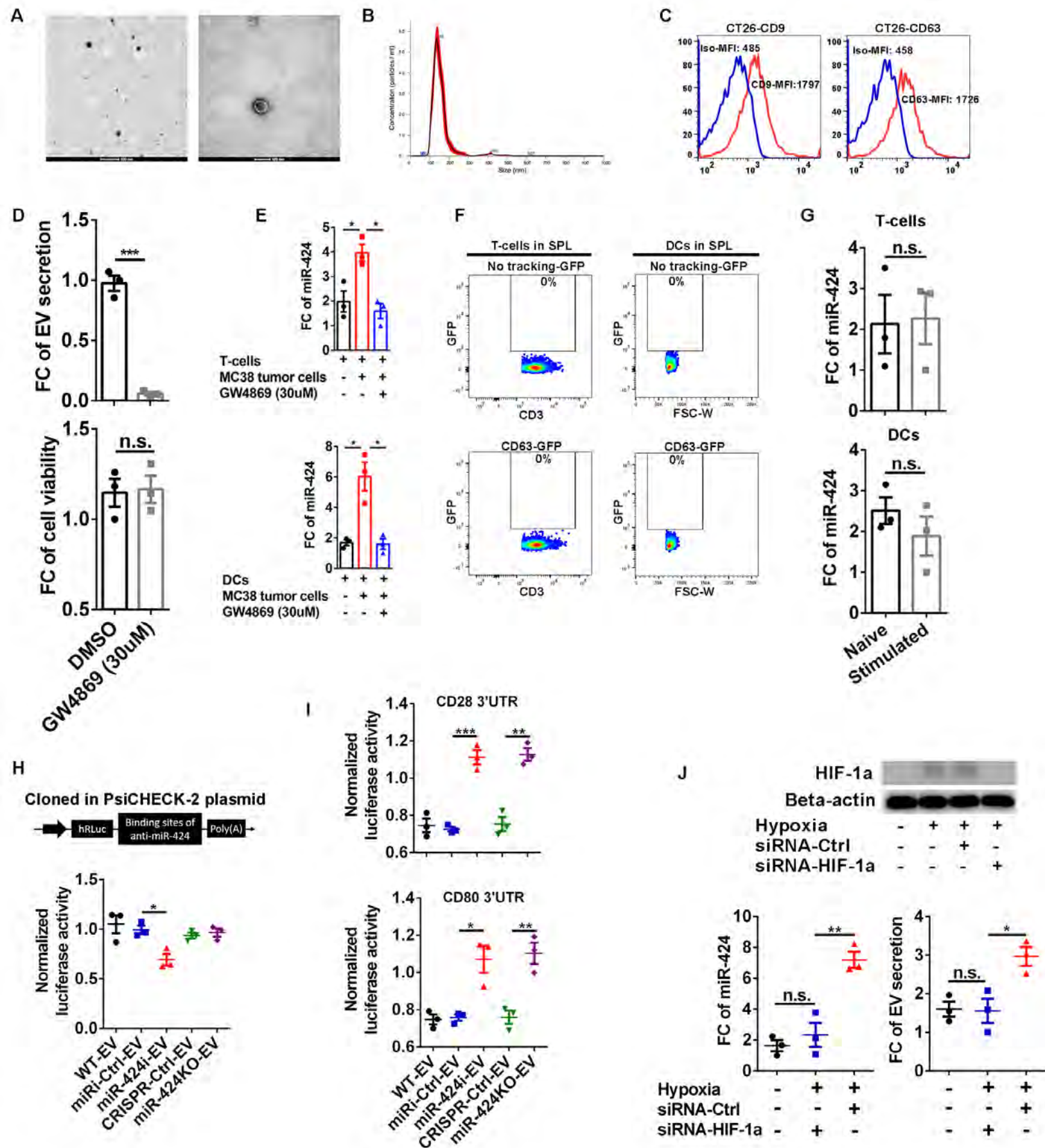


Figure S3. Tumor cells derived EVs (TEVs) transfer miR-424 to immune cells and downregulate CD28 and CD80. Related to Figure 3.

- (A) Transmission electron microscopy was used to capture the image of TEVs (CT26 tumor cell line as a representative).
- (B) Nanosight system was used to measure the size of TEVs (CT26 tumor cell line as a representative).
- (C) CD9 and CD63 protein expression were detected on the surface of TEVs (CT26 tumor cell line as a representative).
- (D) GW4869 reduced tumor cells EVs production but no viability. CT26 tumor cell line as a representative. (Means $\pm$ SEM, n=3, t-test, *** p <0.001)
- (E) Repeat of experiments in Figure 3D in MC38 tumor cell line. (Means $\pm$ SEM, n=3, t-test, * p <0.05)
- (F) T-cells and DCs in the spleen (SPL) of CT26-CD63-GFP tumor bearing mice showed no GFP signal. Related to Figure 3E. (n=3 in each condition)
- (G) Stimulation of naïve mouse T-cells (by CD3/CD28 activation beads) and DCs (by lipopolysaccharide) didn't increase miR-424 expression. (Mean $\pm$ SEM, n=3, t-test, n.s. no significance)
- (H) Complementary sequence of miR-424 inhibitor was cloned into the dual luciferase vector (PsiCHECK-2). The vector was used to confirm the presence of functional miR-424 inhibitors in miR-424i-EVs. (0.05 μ g TEVs were supplied to every 10^4 cells, Mean $\pm$ SEM, n=3, t-test, * p <0.05)
- (I) Dual luciferase assay confirmed functional miR-424 in CT26 TEVs could bind with the 3'UTRs of CD28 and CD80. No functional miR-424 was detected in miR-424i-EV and miR-424KO-EV. (0.05 μ g TEVs were supplied to every 10^4 cells, Mean $\pm$ SEM, n=3, t-test, * p <0.05, ** p <0.01, *** p <0.001)
- (J) HIF-1 α accumulation was induced by hypoxia in HT29 cells. HIF-1 α accumulation led to increased miR-424 expression and EVs production in HT29 cells. (Mean $\pm$ SEM, n=3, t-test, * p <0.05, ** p <0.01, n.s. no significance)

Figure S4

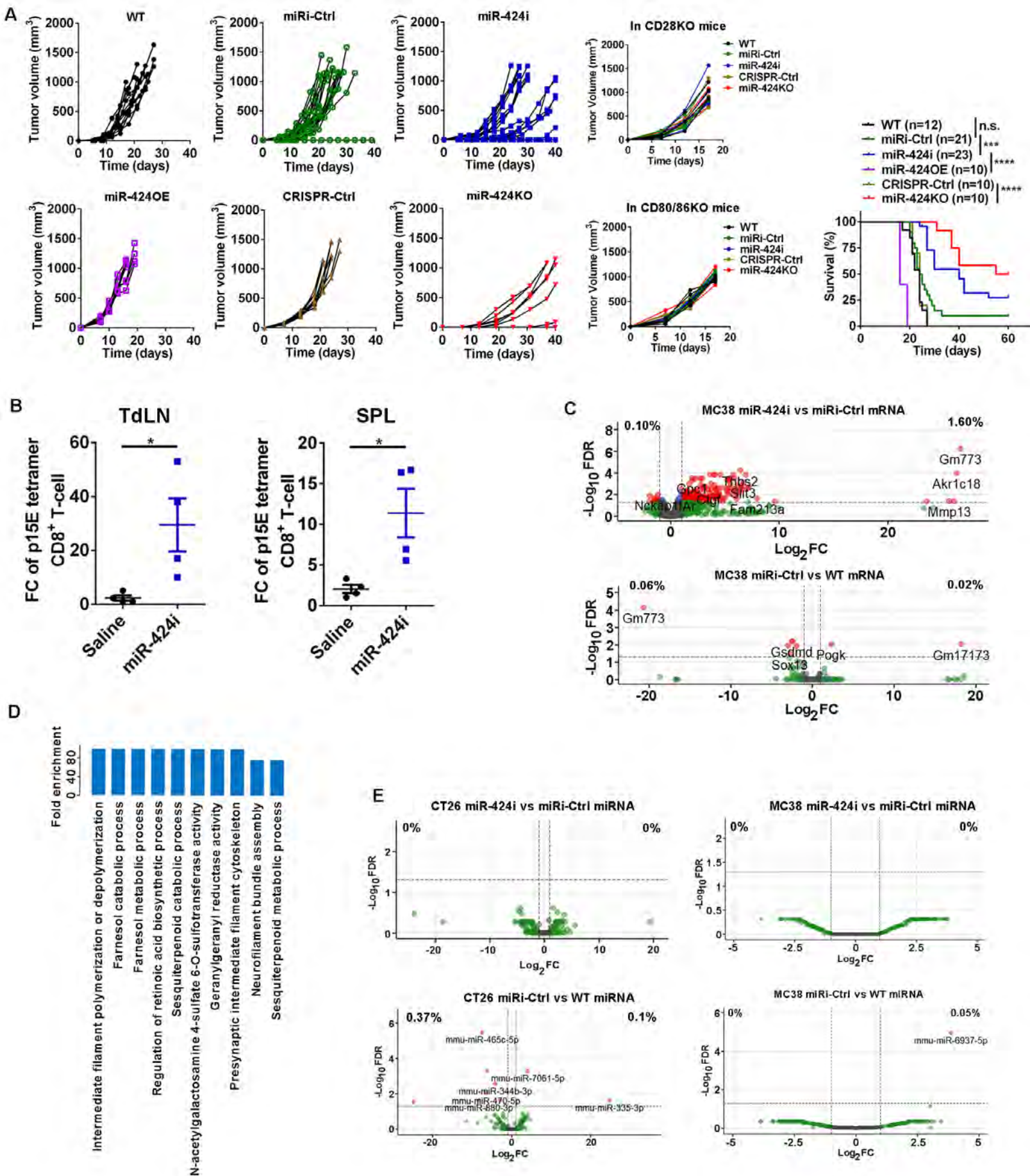


Figure S4. Tumor EVs with functional miR-424 accelerated tumor growth by suppressing antitumor immunity. Related to Figure 4.

(A) Repeat of experiments in Figure 4D with MC38 tumor cell line in WT C57BL/6 mice, *Cd28*^{-/-} mice and *Cd80/86*^{-/-} mice. (n=10-20, Kaplan-Meier method for survival curve, log-rank test for survival analysis, ****p*<0.001, *****p*<0.0001)

(B) Anti-tumor specific immune response was detected in the tumor free mice that were injected with miR-424i MC38 tumor cells. (Means±SEM, n=4, t-test, **p*<0.05)

(C) mRNA-Seq data showed the proportion of differentially expressed genes in MC38 cells with and without functional miR-424.

(D) The Gene Ontology analysis showed biological pathways that were up/down regulated in MC38 cells with and without functional miR-424.

(E) miRNA-Seq data showed the proportion of differentially expressed miRNAs in CT26 and MC38 cells with and without functional miR-424.

Figure S5

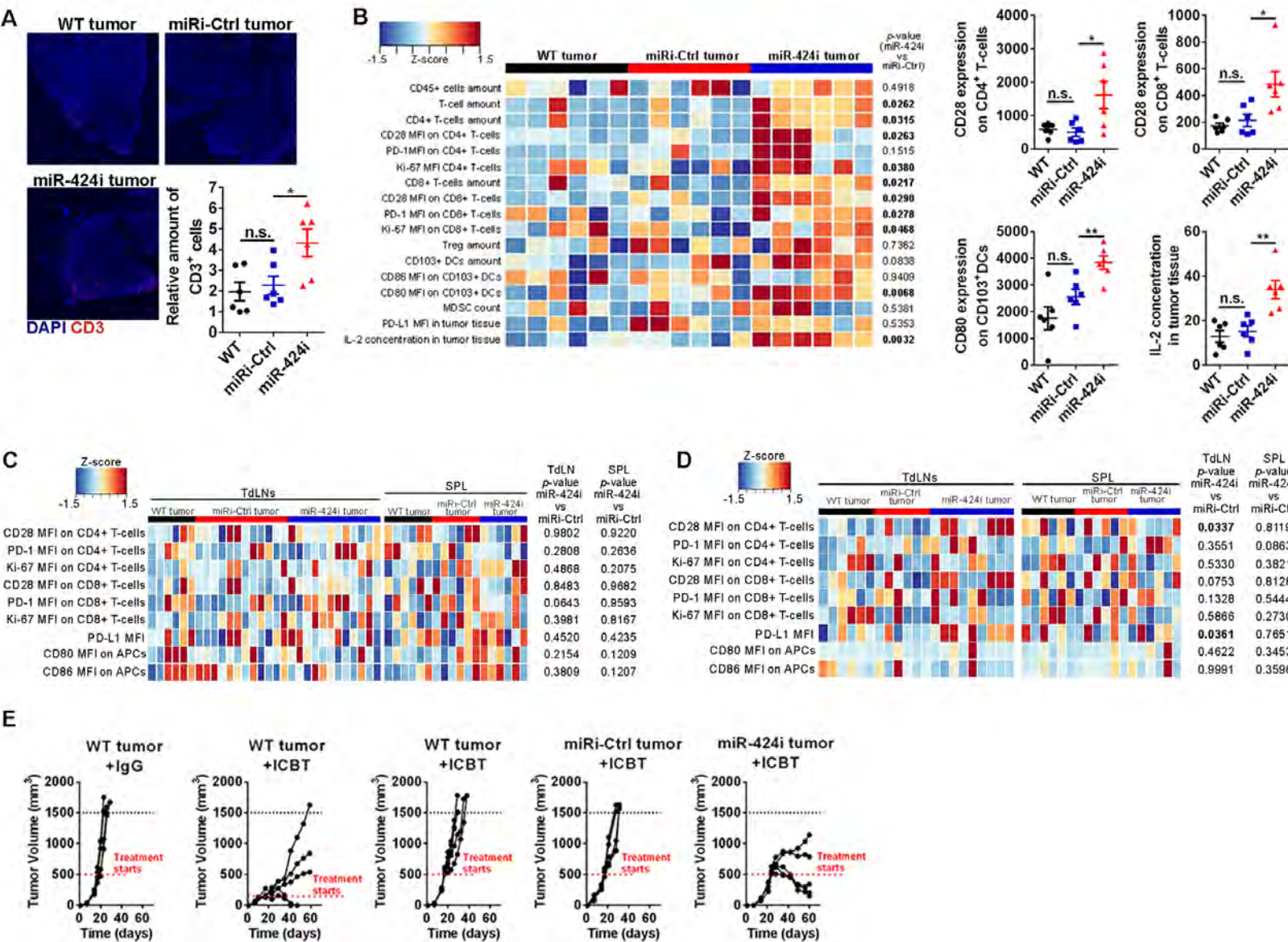


Figure S5. Depletion of functional miR-424 enhanced the costimulatory molecules CD28 and CD80 expression, antitumor immunity, and response to ICBT. Related to Figure 5.

(A) The overall T-cell infiltration in MC38 tumors with (WT, miRi-Ctrl) and without (miR-4242i) functional miR-424. T-cells were observed in both peripheral and central parts of tumors. (Mean±SEM, n=6, t-test, ** $p<0.01$ n.s. no significance)

(B) The anti-tumor immune profiles in MC38 tumors without functional miR-424 and control tumors were shown in the heatmap. Quantitate analysis was shown between miR-424i and miRi-Ctrl groups at the right of heatmap. Right panels: Quantitate analyses of the CD28 expression on tumor infiltrating CD4⁺ and CD8⁺ T-cells, CD80 expression on tumor infiltrating CD103⁺DCs, and the intratumoral IL-2 concentration were shown in all three groups. (Mean±SEM, n=6, t-test, * $p<0.05$, ** $p<0.01$ n.s. no significance)

(C) The immune landscape of TdLNs and SPL in the CT26 (WT, miRi-Ctrl, miR-424i) tumor bearing mice. Quantitate analyses were shown at the right of the heatmap.

(D) The immune landscape of TdLNs and SPL in the MC38 (WT, miRi-Ctrl, miR-424i) tumor bearing mice. Quantitate analyses were shown at the right of the heatmap.

(E) In the CT26 tumor model, ICBT is effective for treating the early stage tumors (volume at around 100mm³) but not for controlling the established tumors (volume at around 500-700mm³). Depleting the tumor cells derived functional miR-424 enhanced the response of established tumor response to ICBT.

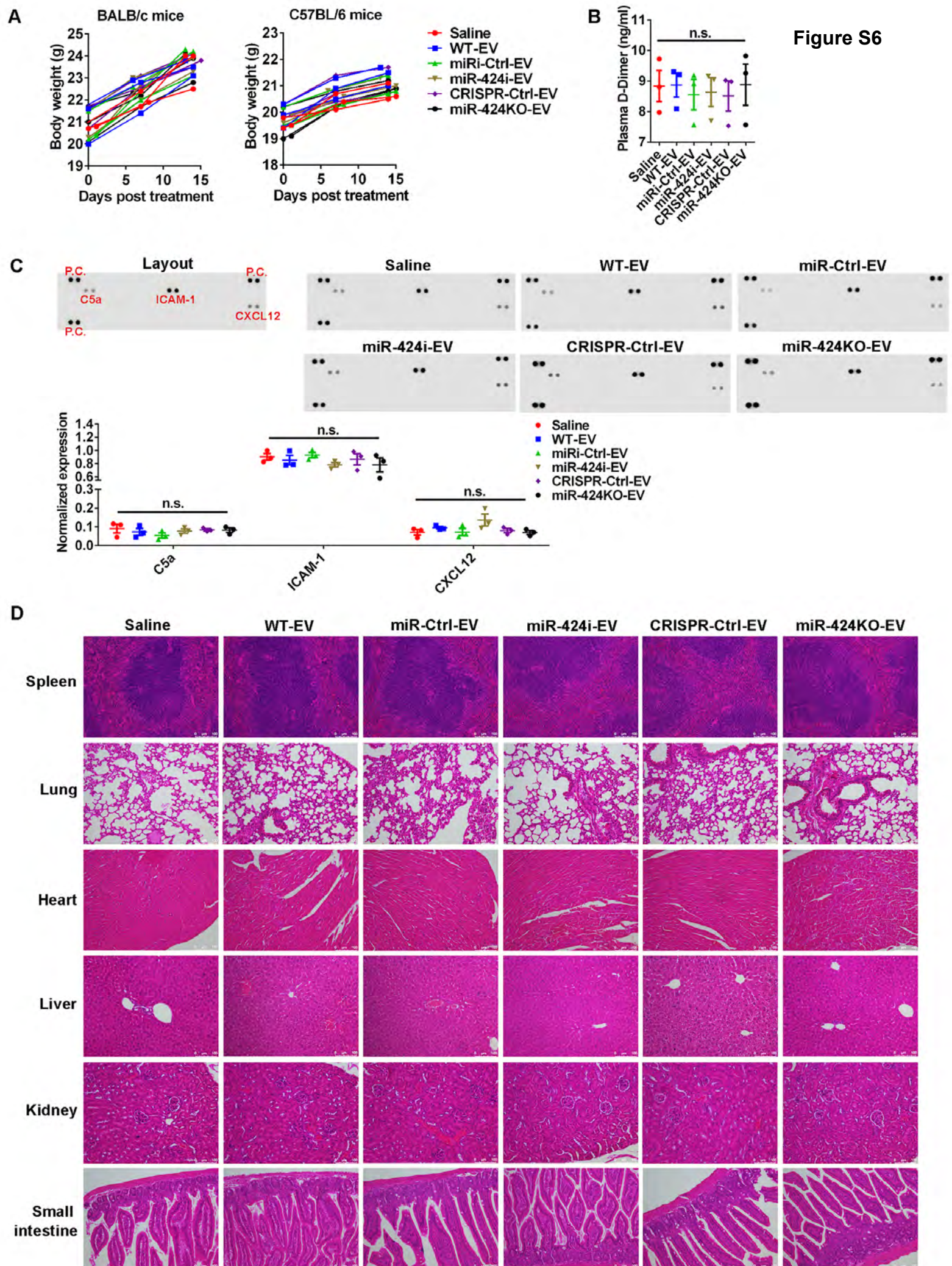


Figure S6. TEVs without functional miR-424 are efficient in stimulating antitumor immune response. Related to Figure 6.

(A) Influence of short-term TEVs injection on the naïve Babl/c mice growth curve was shown (2 doses, 10ug/dose).

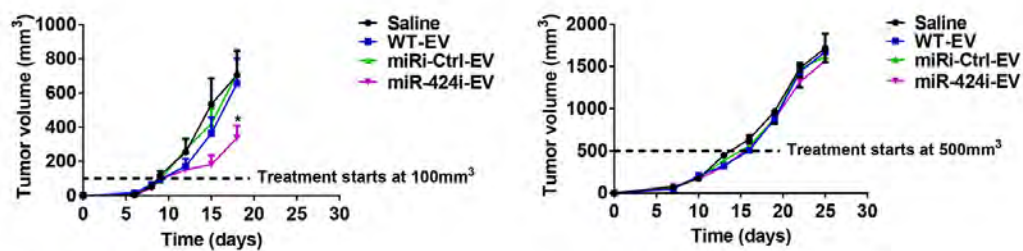
(B) Thrombosis level (indicated by the serum D-dimer level) in naïve Babl/c mice treated by short-term TEVs injection (2 doses, 10ug/dose). (Mean±SEM, n=3, t-test, n.s. no significance)

(C) Serum cytokine levels in naïve Babl/c mice treated by short-term TEVs injection (2 doses, 10ug/dose). The upper panels showed representative data of the cytokine array (n=3). The lower panel showed the quantitative results of C5a, ICAM-1, CXCL12. (Mean±SEM, n=3, t-test, n.s. no significance)

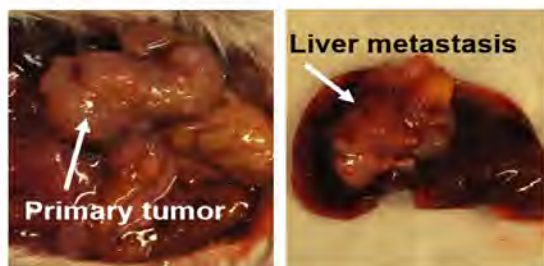
(D) Histological analysis of the major organs (spleen, lung, heart, liver, kidney, and small intestine). Tissue samples were taken from mice treated by short-term TEVs injection (2 doses, 10ug/dose). (n=6, one representative picture from each group)

Figure S7

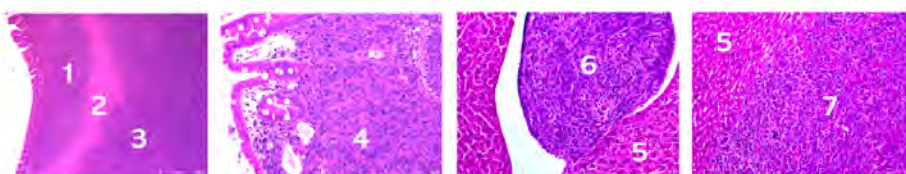
A



B



C



D

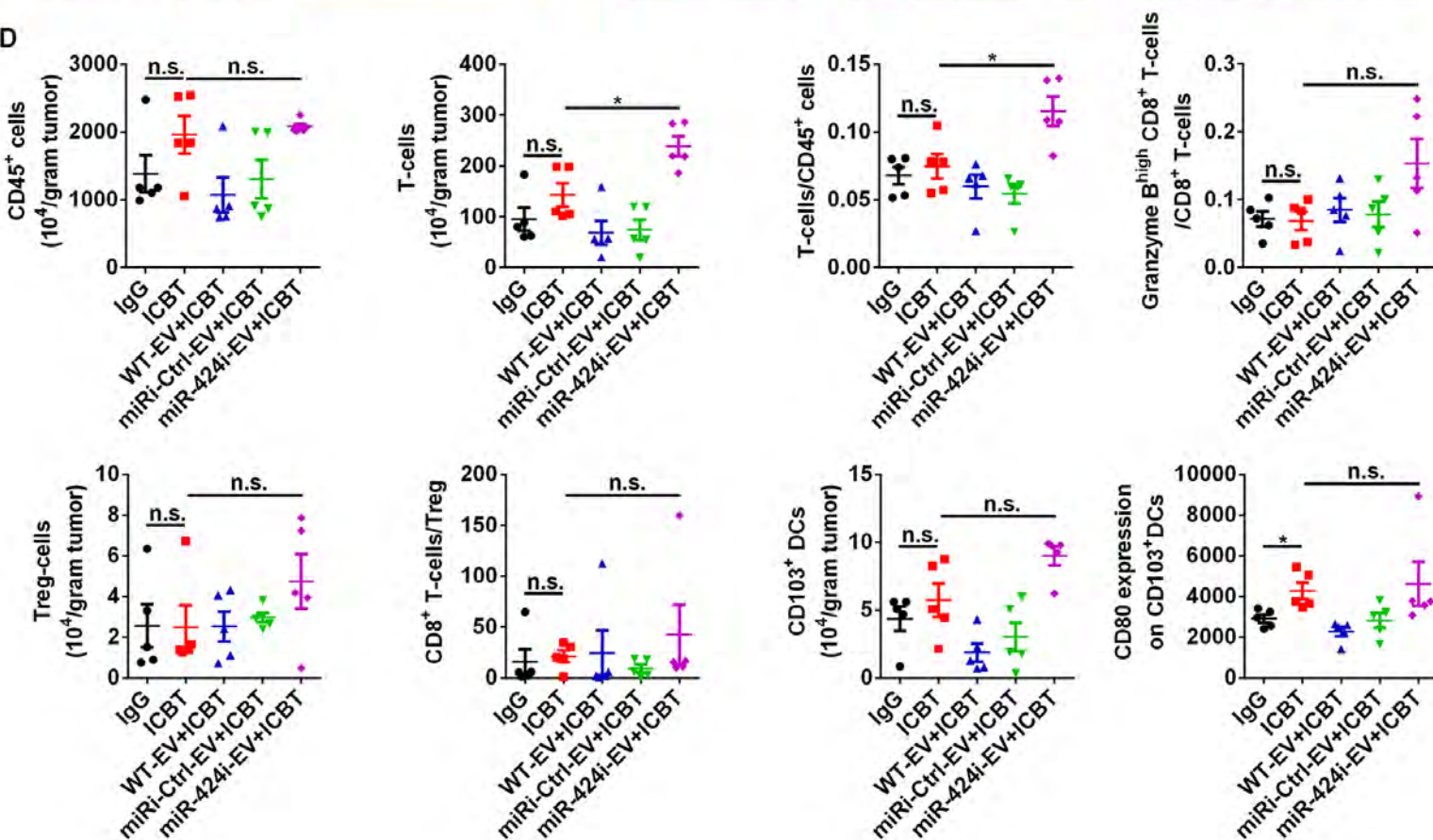


Figure S7. TEVs without miR-424 enhanced the efficacy of ICBT in established orthotopic tumors. Related to Figure 7.

(A) Left: Response of early stage tumors (100-200mm³) to short-term different TEVs injection (2 doses, 10ug/dose). Right: Response of established tumors (500-700mm³) to short-term different TEVs injection (2 doses, 10ug/dose). (Mean±SEM, n=5, one-way ANOVA for multiple groups comparison, * $p<0.05$)

(B) The primary orthotopic cecum tumor and liver metastatic lesions were shown.

(C) The histology of mouse cecum orthotopic tumor was shown. 1: tumor cells in submucosa; 2: tumor cells in muscularis externa; 3: tumor cells outside the colon wall; 4: tumor cells in mucosa; 5: normal liver tissue; 6: tumor cells spreading to the surface of liver; 7: tumor metastasis in the liver.

(D) The supplementary quantitative analysis of figure 7D.