Melanoma-derived extracellular vesicles (EVs) as drivers of immunosuppression

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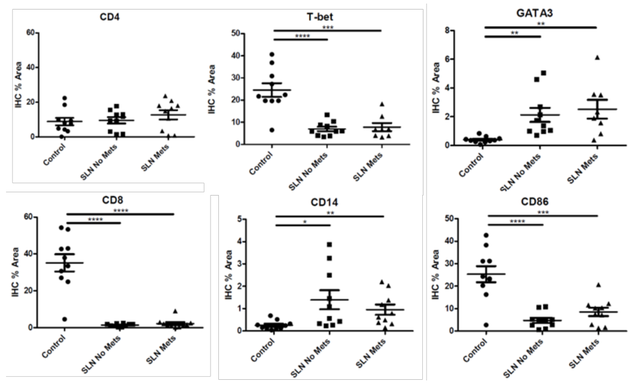
Background

- Evolution of metastatic melanoma from a primary tumor of the skin to widespread dissemination is crucially dependent on early regional lymph node metastases[1].
- Characterization of tumor-draining, sentinel lymph nodes (SLNs) in patients reveals an immunosuppressed state amenable to tumor growth and progression prior to clinical evidence of nodal metastasis[2].
- The observation that regional immunosuppression is independent of nodal involvement suggests the lymphatic microenvironment is altered prior to clinical evidence of metastasis and therefore an alternative mechanism independent of tumor cells is responsible for initiating this process.



Regional immunosuppression precedes nodal metastasis. SLN tissue was collected from patients with melanoma with or without clinical evidence of metastasis and compared to control nodes from patients following prophylactic mastectomies. IHC analysis of immune cell subsets (CD4, CD8, and CD14), DC activation marker (CD86) and T cell transcription factors (T-bet and GATA3) demonstrate an immunosuppressed, Th2 polarized sentinel lymph node profile independent of nodal involvement. (* p<0.05,, ** p<0.01, ***p<0.001, ****p<0.0001). Mansfield et al. (2011). *Mod Path.* 24: 487-494.

Methods

EV isolation and hypoxia: Melanoma (A375, C32TG, SK-MEL-28), Burkitt's lymphoma (Daudi), and triple negative breast cancer (MDA-MB-231) cell lines were incubated for 96 hours with oxygen-exchanging culture flasks (normoxia) or low-oxygen transfer Petaka G3 flasks (Celartia) to induce hypoxia. The vesicles were purified from the supernatants using a water-excluding precipitation (Invitrogen, Life Tech) to enrich for membrane-bound EVs and size distribution and concentration were confirmed using Nanoparticle Tracking Analysis (Nanosight).

Plasma EVs: Human plasma was obtained from patients with melanoma (IRB 430-00) and healthy volunteers. Vesicles were isolated from 1 mL aliquots of plasma using the plasma specific EV isolation kit (Invitrogen, Life Technologies).

Proteomics: EVs were cultured as described above. The final 24 hours of culture were under reduced serum conditions. Purified EVs were lysed, measured for protein content by BCA assay and run on 4-20% TGX Ready gel. Following silver stain, the gel was fractioned, digested and subjected to mass spectrometry analysis at the Mayo Clinic Proteomics Core. Peptide results were matched to the human Swissprot protein database.

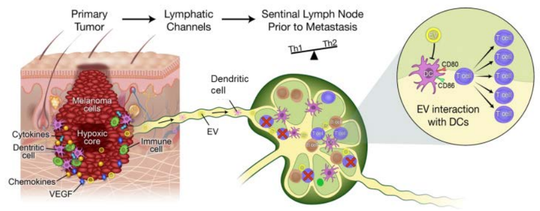
Co-culture: CD14+ monocytes were negatively selected (Miltenyi Biotech) from the PBMCs of healthy human donors. The monocytes were cultured with GM-CSF (10 ng/mL) and IL-4 (1 ng/mL) for 5 days in the presence or absence of SK-MEL-28 derived EVs, plasma-derived EVs or recombinant proteins S100A8 (10ug/mL), Annexin A1 (10ug/mL), and ICAM1 (10ug/mL). The DCs were matured with CD40L (0.01µg/mL), for 2 days. Monocyte and dendritic cell surface markers were assessed by flow cytometry on Days 5 and 7 using surface markers CD14, HLA-DR, HLA-ABC CD40, CD11c, CD86, PDL1 and CCR7 (BD Pharmingen). Statistical significance was determined using two tailed t- tests (p<0.05).

MLR: CD40L matured dendritic cells cultured in the presence or absence of tumor-derived EVs were irradiated and cultured together with haplotype-mismatched CD4+ T cells isolated from a healthy human donor for 5 days. CD4+ T cell proliferation was measured by radioactive thymidine incorporation.

Lymphatic Channels: Afferent lymphatic channels were excised from patients with melanoma undergoing sentinel lymph node biopsy, as is standard of care (IRB 10-000806). Lymphatic fluid was perfused from the channel and EVs were isolated from the fluid utilizing ultracentrifugation and visualized by Nanosight.

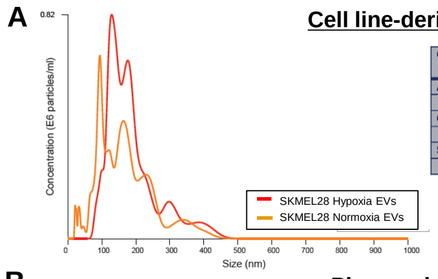
Objectives

In the current study, we evaluated melanoma-derived EVs for their potential to polarize immunity towards an immunosuppressive, tumor-promoting state.

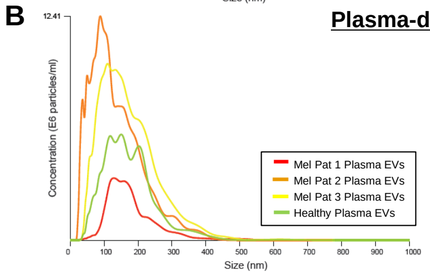


Proposed Model: Hypoxia-induced melanoma EVs are secreted from the primary tumor, navigate the lymphatics and enter sentinel lymph nodes to create an immunosuppressed microenvironment amenable for melanoma cell survival.

Characterization of EVs



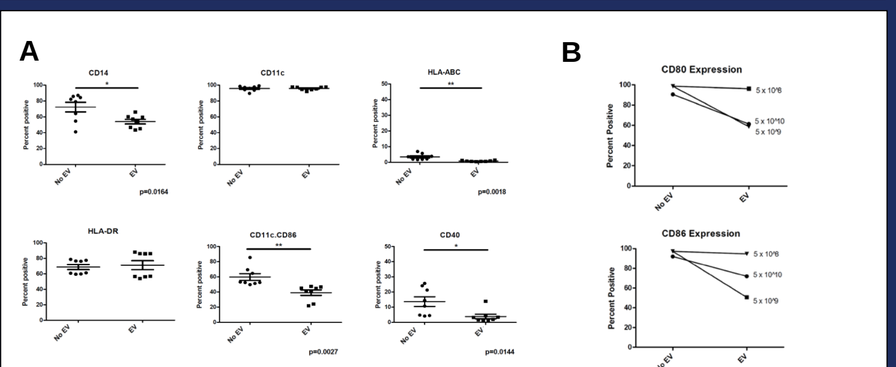
Cell Line	Culture Conditions	Particle Size (nm)	Particle concentration (particles/mL)
A375	Normoxia	164.6 ± 0.73	7.2×10^7
	Hypoxia	280.6 ± 2.16	2.16×10^8
C32TG	Normoxia	134.6 ± 15.01	4.2×10^7
	Hypoxia	181.3 ± 11.93	1.03×10^8
SKMEL28	Normoxia	153 ± 4.35	7.5×10^7
	Hypoxia	165 ± 15.87	1.06×10^9

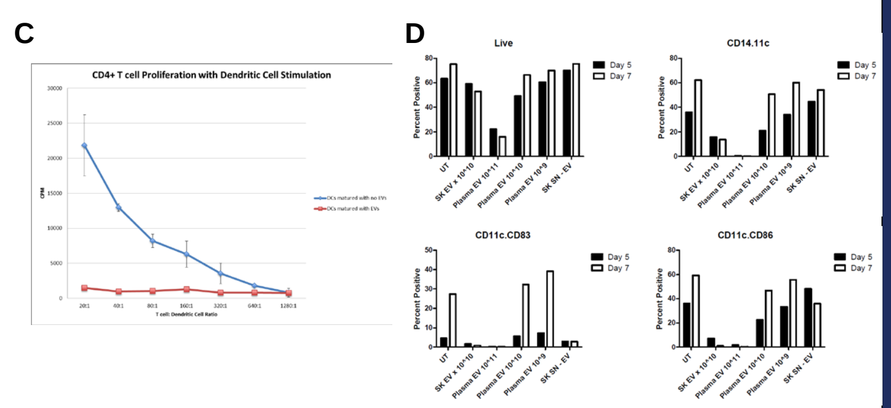


Patient cohort (plasma source)	Particle Concentration (particles/mL)
Melanoma Patient 1	4.49×10^{11}
Melanoma Patient 2	18.09×10^{11}
Melanoma Patient 3	17.68×10^{11}
Healthy	9.86×10^{11}

Validation of EV isolation by Nanotracking Analysis. Representative analysis of size distribution and EV quantification of cell-line derived (A) and plasma-derived (B) EVs utilizing Nanoparticle Tracking analysis (Nanosight).

EVs as Immune Modulators





Melanoma EVs inhibit DC maturation and activation of T cell proliferation *in vitro.* (A) Surface expression of DC phenotypic and maturation markers for CD14+ monocytes matured with CD40L in the presence or absence of melanoma (SKMEL28) EVs. (n=9, *=p<0.05, **=p<0.01). (B) CD80 and CD86 expression in matured DCs following culture with varying doses of SKMEL28 EVs. (C) Mixed lymphocyte reaction assessing T cell proliferation in response to irradiated DCs matured in the presence or absence of melanoma EVs. (D) Surface expression of DC phenotypic markers following culture in the presence or absence of SKMEL28 or plasma-derived EVs.

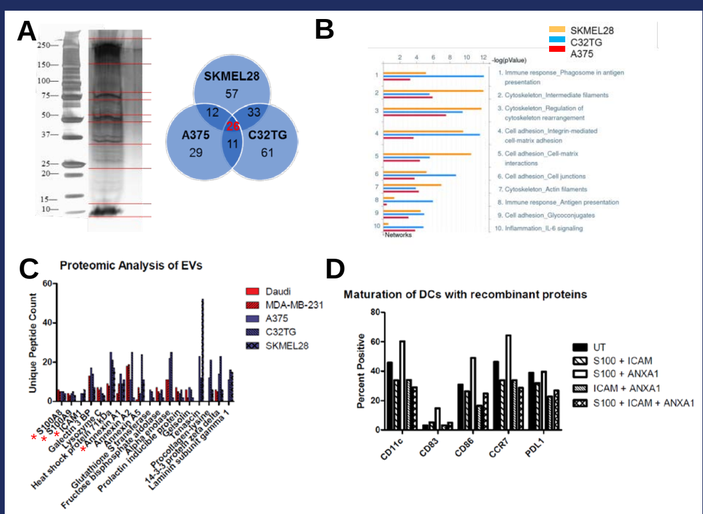
References and Acknowledgements

1. Grotz et al (2012). Regional lymphatic immunity in melanoma. *Melanoma Research*. 22(1):9-18.
2. Mansfield et al. (2011). Regional immunity in melanoma: immunosuppressive changes precede nodal metastasis. *Mod Path.* 24: 487-494.
3. Nevala et al. (2009). Evidence of systemic Th2-driven *Clin Cancer Res* 2009;15:1931-1939.

Mass spectrometry analysis was performed in collaboration with the Mayo Clinic Medical Genome Facility Proteomics Core.

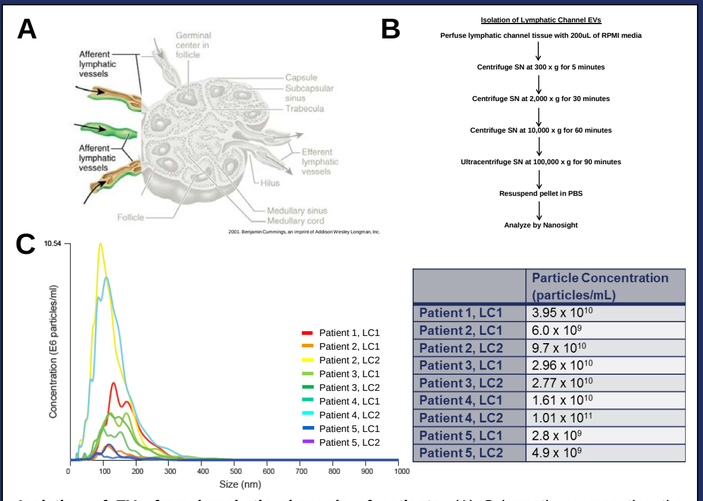
Lymph node and lymphatic channel collection from patients is currently being done in collaboration with James Jakub, MD and the Mayo Clinic Department of Surgery (IRB #10-000806).

EV Proteomics



Proteomic analysis of EV content. (A) Following silver stain, SK-MEL-28 derived EV proteins were visualized and gel fractions (in red) were analyzed by mass spectrometry; unique protein cargo was found in the EVs of each cell line with 26 proteins overlapping. The overlapping proteins in melanoma EVs were analyzed by (B) Pathway enrichment and (C) compared to non-melanoma cell lines (red). Asterisks indicate protein cargo with known effects on dendritic cell biology. (D) Phenotypic surface marker expression of CD14+ monocytes matured to DCs *in vitro* with CD40L in the presence or absence of recombinant proteins S100A8, ICAM1 and Annexin A1.

Lymphatic EVs in patients



	Particle Concentration (particles/mL)
Patient 1, LC1	3.95×10^{10}
Patient 2, LC1	6.0×10^9
Patient 2, LC2	9.7×10^{10}
Patient 3, LC1	2.96×10^{10}
Patient 3, LC2	2.77×10^{10}
Patient 4, LC1	1.61×10^{10}
Patient 4, LC2	1.01×10^{11}
Patient 5, LC1	2.8×10^9
Patient 5, LC2	4.9×10^9

Isolation of EVs from lymphatic channels of patients. (A) Schematic representing the anatomical structure of a human lymph node, highlighting the afferent, lymphatic channels. (B) Summary of the EV isolation protocol. (C) Size distribution and quantification of EVs isolated from the lymphatic channel fluid of patients with melanoma undergoing sentinel lymph node biopsy.

Conclusion

- Under hypoxic conditions, increased EV production is observed in melanoma cell lines. EVs isolated from human plasma showed increased EV yield compared to cell line EVs irrespective of the presence of disease.
- Melanoma cell line EVs inhibit DC maturation and functional activation of T cells in a dose-dependent manner. Plasma-derived EVs are unable to inhibit DC maturation, suggesting these EVs are derived from a heterogeneous population of malignant and normal cells.
- Proteomic analysis of melanoma cell line EVs has identified candidate immunological and metastasis associated cargo. Culturing DCs with a subset of this cargo is insufficient for inhibiting DC maturation suggesting critical, synergistic interactions among the cargo proteins are required.
- EVs can be isolated from the lymph of afferent lymphatic channels obtained from patients undergoing sentinel lymph node biopsy. We are currently exploring EVs as a viable communication mechanism between the primary tumor and sentinel lymph node.