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Melanoma Exosome Induction of Endothelial Cell GM-CSF in Pre-metastatic Lymph Nodes may Result in Different M1 and M2 Macrophage Mediated Angiogenic Processes

Joshua L. Hood, M.D., Ph.D.

University of Louisville, Department of Pharmacology and Toxicology and the James Graham Brown Cancer Center, Clinical and Translational Research Building, 505 South Hancock Street, Louisville, KY 40202, Phone: 1-502-852-8411, Fax: 1-502-852-3661

Abstract

Angiogenesis is a key process in the preparation of lymph nodes for melanoma metastasis. Granulocyte macrophage colony stimulating factor (GM-CSF) induces hypoxia inducible factor 1 alpha (HIF-1 α) in M1 or HIF-2 α in M2 polarized macrophages. HIF-1 α promotes neoangiogenesis while HIF-2 α facilitates morphogenic normalization of neovasculature.

Melanoma exosomes induce GM-CSF expression by endothelial cells *in vitro* and HIF-1 α expression in pre-metastatic lymph nodes *in vivo*. This suggest a relationship between melanoma exosome induced endothelial GM-CSF and macrophage mediated angiogenesis in lymph nodes. Theoretically, induction of endothelial cell derived GM-CSF by melanoma exosomes mediates different angiogenic functions in pre-metastatic lymph nodes depending on subcapsular sinus (SCS) macrophage polarity. To explore this hypothesis, experiments utilizing melanoma exosomes in a lymph node model are outlined. Despite their opposing immune functions, indirect melanoma exosome stimulation of M1 or M2 SCS macrophages via endothelial derived GM-CSF in lymph nodes may induce different although complementary pro-tumor angiogenic processes.

Introduction

Within tumor draining lymph nodes, melanoma derived soluble mediators such as vascular endothelial growth factor (VEGF) increase angiogenesis enabling tumor growth and survival (1). Melanoma derived cytokines mediate immune suppression (2).

Previously, Mansfield et al. demonstrated that the microenvironment of dormant human lymph nodes is Th1 polarized (3). Th1 lymphocytes release cytokines such as IFN- γ which polarize M ϕ s toward an anti-tumor M1 phenotype (4). Recent evidence demonstrates Th2 inflammatory polarization within melanoma draining sentinel lymph nodes (5). This is true

Corresponding Author: joshua.hood@louisville.edu.

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for sentinel lymph nodes obtained from melanoma patients with or without metastasis (3). Th2 cytokines such as interleukin 4 (IL-4), IL-5, and IL-10 polarize macrophages (Mφs) toward an immunosuppressive M2 phenotype in melanoma microenvironments (6). Tumor draining factors are thought to be responsible for Th2 polarization in lymph nodes in the absence of lymph node associated melanoma cells (3).

Melanoma cells also produce exosomes. Exosomes are natural extracellular nanovesicles approximating 30-200 nm in size (7-10). Similar to soluble mediators, tumor exosomes can transmit pathogenic processes (11, 12). Melanoma exosomes can re-program bone marrow progenitor cells toward a pro-vascular phenotype (13) or directly induce angiogenesis (11, 14). They also contain pro-angiogenic and immunomodulatory factors (14) and can prepare lymph nodes for tumor metastasis (15).

One immunomodulatory strategy to treat melanoma is the administration of granulocyte-macrophage colony stimulating factor (GM-CSF) (6). GM-CSF has long been known to facilitate proliferation, expansion and survival of granulocytes and Mφs. However, in recent years it has also been shown to increase maturation and expansion of dendritic cells (DCs). DCs present melanoma antigens resulting in activation of effector T lymphocytes and subsequent anti-tumor immune responses. In pre-clinical trials of patients with advanced melanoma, GM-CSF monotherapy was efficacious in inhibiting melanoma growth (16). However, in prospective, randomized clinical trials, the efficacy of GM-CSF monotherapy was inconsistent. Conflicting findings suggested that GM-CSF might serve as a regulatory cytokine promoting both poorly understood pro- and anti- tumor immune responses (6).

In addition to its immunological roles, GM-CSF can induce angiogenesis via stimulation of endothelial cells and/or activation of Mφs to produce pro-angiogenic factors (17). Given the interconnectedness between cytokine mediated immunological and angiogenic processes (17), the paradoxical nature of GM-CSF function may extend to its role in angiogenesis as well.

Presentation of the Hypothesis

Within the lymph node, CD169+ subcapsular sinus (SCS) Mφs limit viral or tumor cell dissemination (18). They process tumor draining antigens and activate tumor antigen specific lymphocyte responses. In regional lymph nodes obtained from melanoma patients a higher cell density of CD169+ SCS Mφs is associated with longer overall patient survival (18). Given that melanoma exosomes contain a wealth of tumor source cell antigens and are structurally similar to viruses, it is reasonable that they should be collected by SCS Mφs in tumor draining lymph nodes. This was recently demonstrated by Pucci et al. in mice who showed that CD169+ SCS Mφs serve as protective gatekeepers by physically preventing B16-F10 melanoma exosomes follicular access to B lymphocytes to facilitate pro-tumor humoral immunity (19). Interestingly, the authors found that CD169+ SCS Mφ density in nodes did not keep pace with tumor draining lymph node enlargement. If translated to a clinical scenario, based on the findings of Saito et al. (18), such patients would be expected to have a poor survival outcome.

Melanoma exosome modulation of CD169+ SCS M ϕ function was not reported by Pucci et al. to involve the mechanism of horizontal transfer (19). Nevertheless, the lack of expansion of CD169+ SCS M ϕ s observed by Pucci et al. is consistent with immunosuppressive signaling effects observed for melanoma exosome interactions with other types of immune cells (20). Melanoma exosomes have been shown to suppress natural killer and cytotoxic CD8+ lymphocytes (20). They can also induce myeloid derived suppressor cells MDSCs (2) which can anergize anti-tumor CD8+ lymphocytes (21, 22). MDSCs in turn promote M2 M ϕ polarization and recruit pro-tumor regulatory T cells (6).

Increased GM-CSF and the M2 polarizing cytokine IL-10 are both found in melanoma draining sentinel lymph nodes (23). Melanoma cell derived GM-CSF also increases monocyte production of tumor necrosis factor alpha (TNF- α) (24). TNF- α can increase hypoxia inducible factor 1 alpha (HIF-1 α) production by M ϕ s (25). HIF-1 α is activated in M1 M ϕ s whereas HIF-2 α is activated in M2 M ϕ s (25, 26).

The balance between M1 and M2 M ϕ phenotypes and tumor angiogenesis is complex. HIF-1 α activation in M1 M ϕ s results in expression of VEGF and blood vessel growth (25). Other studies demonstrate that HIF-2 α is also pro-angiogenic and increases morphogenic normalization and organization of tumor supportive neovasculature (27, 28). This improves the vascular patency and oxygen delivery efficiency of tumor blood vessels.

The potential range of pro-angiogenic activities permitted by HIF-1 α and HIF-2 α working in concert may greatly facilitate tumor survival. In one prognostic study, pathologic features of tumor aggressiveness were more correlated to HIF-2 α expression than HIF-1 α (29). Further, treating HIF-1 α knockout or HIF-2 α siRNA depleted M ϕ s with GM-CSF under hypoxic conditions, as might be encountered in a tumor microenvironment, revealed distinct pro-angiogenic roles for HIF-1 α and HIF-2 α in M ϕ s (30). HIF-1 α drives VEGF expression whereas HIF-2 α drives soluble VEGFR-1 (sVEGFR-1) expression by M ϕ s. Extrapolation of the findings with consideration to M ϕ polarity implies that the angiogenic effect of GM-CSF acting on M1 M ϕ s expressing HIF-1 α will be different than the effect of GM-CSF acting on M2 M ϕ s expressing HIF-2 α . It is known that M ϕ derived VEGFR-1 can associate with and attenuate the activity of M ϕ derived VEGF (31). It follows that sVEGFR-1 derived from M2 M ϕ s can associate with and attenuate the activity of VEGF derived from M1 M ϕ s.

Earlier data demonstrated that melanoma exosomes induce GM-CSF expression by 3D cultured vascular endothelial cells (VECs) *in vitro* (11) and HIF-1 α expression in pre-metastatic lymph nodes *in vivo* (15). Collectively, these exosome data and the previously discussed GM-CSF and M ϕ data support the hypothesis that induction of endothelial cell derived GM-CSF by melanoma exosomes mediates different angiogenic functions in pre-metastatic lymph nodes depending on SCS M ϕ polarity (**Fig. 1**).

Evaluation of the Hypothesis

Melanoma exosomes naturally home to the subcapsular sinus (SCS) of lymph nodes (15, 32). In nodes, SCS resident CD169+ M ϕ s participate in tumor mediated immunity and can capture B cell exosomes (33) and melanoma exosomes (19). Given their exposure to tumor

draining factors such as cytokines, growth factors and exosomes, SCS resident CD169+ M ϕ s would likely be susceptible to melanoma driven M1 or M2 polarization processes. They are also likely to be exposed to lymphatic VEC derived GM-CSF induced by melanoma exosomes. SCS resident CD169+ M ϕ s are thus a good candidate M ϕ population for testing the hypothesis.

From previous data, it is known that melanoma exosomes, in the absence of tumor cells, induce HIF-1 α in wildtype c57BL/6 mouse lymph nodes (15). While this suggest a pre-existent baseline SCS M1 polarity in the lymph nodes in non-tumor bearing mice, it is unknown the extent to which HIF-2 α is also upregulated. The presence of HIF-2 α would suggest some degree of co-existent SCS M2 polarity.

With this in mind, reducible *ex vivo*, mouse endothelial and M ϕ experiments can be performed to assess melanoma exosome mediated induction of lymph node endothelial GM-CSF in the context of CD169+ SCS resident M ϕ polarity using our exosome lymph node trafficking model (15, 32). In the model, mouse popliteal lymph node VECs treated with melanoma exosomes (fluorescent red) *in vivo* can be isolated by fluorescent activated cell sorting (FACS) for VCAM-1 positive, lymphatic vessel endothelial receptor 1 (LYVE-1) negative endothelial and CD169+ SCS resident M ϕ s. LYVE-1 marker negativity is used to differentiate VECs from lymphatic endothelial cells (LECs) that are LYVE-1+. Alternatively or coincidentally, melanoma exosomes might also induce GM-CSF production by LECs as well as VECs. This possibility can be investigated as well using FACS. Once sorted, real time reverse transcriptase (RT²) polymerase chain reaction (pcr) can be used to quantify expression of GM-CSF mRNA in endothelial cells and VEGF and/or sVEGFR-1 mRNA expression in M ϕ s. Similar FACS and RT² pcr based evaluations can be performed on melanoma patient derived negative and positive sentinel lymph nodes at different metastatic stages.

Complementary experiments crossing mice expressing inducible Cre-recombinase in VECs or LECs with mice expressing a floxed allele for GM-CSF with a downstream fluorescent reporter could further differentiate whether melanoma exosomes specifically induce GM-CSF in VECs, LECs or both in lymph node investigations performed in the offspring. A similar Cre-recombinase strategy could be used for macrophage selective deletion of HIF-1 α or HIF-2 α as reported by other investigators (34, 35) to further determine whether mRNA expression of VEGF and sVEGFR-1 are specific to FACS sorted SCS M1 and M2 polarized M ϕ s respectively.

Categorizing M1 and M2 M ϕ s derived from the lymph nodes requires gold standard functional markers given the potential for intermediate phenotypes (36). Some of these include upregulation of inducible nitrogen oxide synthase (iNOS) for M1 and arginase 1 (Arg-1) for M2. Commonly employed cytokine markers include TNF- α and IL-1 β for M1 or IL-10 and TGF- β for M2 (36).

Further subdivisions exist within the general M2 M ϕ category as outlined in an excellent recent review (37). Definitive M2 subclassification remains a work in progress given the overlap between markers of M ϕ polarity. Nevertheless, a reasonable scheme divides M2

macrophages into M2a, M2b, M2c and M2d subclasses. M2a M ϕ 's are typically induced by IL-4 whereas M2b's are polarized by immune complexes and lipopolysaccharide (LPS). M2c's are activated by glucocorticoids, transforming growth factor beta (TGF- β) and IL-10. IL-6 and adenosine drive M2d polarization.

In general, IL-10 production is common for all M2 subclasses (37). Other markers such as CD163 and CD206 are shared by M2a and M2c subclasses. TGF- β is an activation marker in M2a, M2c and M2d subclasses. TNF- α is induced in M2b and M2d's. Major histocompatibility complex II (MHC-II) expression is upregulated in M2a and M2b's. More specific M2 subclass markers include Arg-1, Chil3 (chitinase-like 3 or Ym1), resistin-like molecule alpha (RELM- α or FIZZ1), transglutaminase 2 (TGM-2) and interleukin 1 receptor antagonist (IL-1ra) for M2a's. M2b's characteristically express IL-1, IL-6 and CD86. M2c's upregulate c-met proto-oncogene tyrosine kinase (MERTK) and extracellular matrix (ECM) while M2d's express VEGF-A and IL-12. Such M2 subclass markers can be used to further define the SCS resident M2 M ϕ s subclass that upregulates HIF-2 α in lymph nodes treated with melanoma exosomes. Polarization markers for pooled lymph node M ϕ s isolated by FACS can be assessed by RT² pcr for mRNA expression.

To evaluate angiogenic differences between GM-CSF treated M1 versus M2 M ϕ s *ex vivo*, mouse CD169+ SCS or negative selection enriched human peripheral blood monocytes can be differentiated into M1 or M2 M ϕ phenotypes using M1 (lipopolysaccharide, IFN- γ) and M2 (IL-4, IL-10) soluble mediators (38). For simplicity, M ϕ differentiation might be performed in co-culture with compatible mouse 2F2B (11) or human (HUVEC) endothelial cells and melanoma exosomes. The setup approximates the lymph node situation *in vivo* where melanoma exosomes, endothelial cells, M ϕ s and M1 and/or M2 polarizing cytokines would be present simultaneously. A 48 hour culture time point might be used initially based on previous *in vivo* lymph node studies (15). Analysis of post culture supernatants using cytokine ELISAs and Western blots can determine the presence of GM-CSF, VEGF and sVEGFR-1. FACS using fluorescent lipophilic cell tracers (15) and RT² pcr for mRNA might further be used to determine whether the soluble mediators are derived from endothelial or M ϕ cells. A 3D endothelial culture variation of the experimental setup (11) might serve to reveal differences in endothelial growth and morphology generated by M1 versus M2 M ϕ s.

Other experiments can be performed *in vivo* using the lymph node model system. Depending on whether SCS CD169+ M ϕ s exhibit baseline M1 polarity following melanoma exosome lymph node treatment, testing the effect of melanoma exosome induced endothelial GM-CSF on M2 polarized SCS CD169+ M ϕ s may require pretreatment of lymph nodes with Th2 polarizing cytokines such as IL-10 prior to challenge with melanoma exosomes. Th1 polarizing cytokines (IFN- γ) may also be required to skew SCS CD169+ M ϕ s toward the M1 phenotype if minimal SCS CD169+ M ϕ s M1 polarity is observed in lymph nodes post melanoma exosome treatment.

Variations on the lymph node experiments will necessarily involve evaluating different exosome doses and repeat exosome dosing over differing time intervals. Melanoma exosomes themselves may directly have an effect on the polarization of lymph node SCS

CD169+ Mφs. It could be that repeat melanoma exosome dosing over time slowly polarizes the lymph node Mφ population from M1 to M2. This could in turn effect the ratio of VEGF to sVEGFR-1 present in the nodes mediated indirectly by melanoma exosome induction of nodal endothelial cells. To efficiently detect time dependent associations between melanoma exosome associations with VECs, LECs or SCS Mφs in mouse lymph nodes, immunofluorescent imaging of lymph node cross sections can be employed. Sections can be prepared from lymph nodes obtained post exosome administration at different hourly time points. This could serve to elucidate the time course of fluorescent melanoma exosome uptake by endothelial cells and SCS Mφs in lymph nodes using cell specific markers.

Additional experimentation will also be required to determine which melanoma exosome component(s) are responsible for induction of endothelial GM-CSF. It's possible that melanoma exosomes carry translatable GM-CSF mRNA which could be detected by RT² pcr given that melanoma cells themselves produce GM-CSF (24). If true, one implication is that continuous bombardment of tumor draining lymph node endothelial cells by melanoma exosomes may result in consistent delivery to and expression of exosomal GM-CSF shuttle mRNA by endothelial cells. During melanoma pathogenesis, this could result in a kind of cumulative effect. The concentration of melanoma exosomes in tumor draining lymph nodes would be expected to rise with increased tumor growth resulting in a corresponding rise in endothelial cell production of GM-CSF in nodes.

Testing the ability of melanoma exosome associated GM-CSF mRNA to induce GM-CSF production by endothelial cells might be performed by depleting melanoma exosome associated GM-CSF mRNA. Exosomal RNA might be removed via electroextraction (39) or melanoma exosomes obtained from GM-CSF siRNA treated melanoma cells for experimentation.

Similar exosomal RNA depletion experiments might be performed to test the intriguing possibility that melanoma exosomes carry translatable HIF-1α or HIF-2α shuttle mRNA. Our previous research indicated that melanoma exosomes induce HIF-1α in pre-metastatic lymph nodes (15). This could be a result of melanoma exosomes having acted directly on SCS M1 Mφs to induce HIF-1α. Melanoma exosomes may also carry HIF-2α mRNA and directly induce SCS M2 Mφs expression of HIF-2α.

Additional testing of exosomal shuttle miRNA(s) and/or receptor signaling pathways might be necessary as well. A recent study demonstrated that hypoxia resistant multiple myeloma (HRMM) derived exosomes contain miR-135b which can directly suppress factor-inhibiting HIF-1α (FIH-1) (40). HRMM exosomes were shown to enhance endothelial tubule formation under hypoxia via utilization of the HIF-1α/FIH-1 signaling pathway. Melanoma exosomes sourced from patient biofluids may contain similar miR's that induce HIF-1α or HIF-2α function in SCS Mφs.

Supplementary experiments will be necessary to determine the contribution of other cellular sources of GM-CSF and soluble GM-CSF drivers in lymph nodes besides endothelial cells and melanoma exosomes. Other potential cellular sources of GM-CSF that could be induced by melanoma exosomes in lymph nodes include Mφs themselves, T lymphocytes, stromal

cells and DCs (41). These cell types might also be isolated at different time points post melanoma exosome treatment by FACS for *ex vivo* experimentation or associations with fluorescent melanoma exosomes determined using immunofluorescent staining of lymph node cross sections as described above for VECs and LECs.

Soluble drivers of GM-CSF production include IL-1, IL-1 β , IL-23 and TNF- α . Melanoma cells can produce IL-1 β , IL-10, TNF- α and GM-CSF (42). Melanoma conditioned media may therefore contain these soluble mediators which could influence SCS M ϕ polarity and subsequent induction of HIF-1 α or HIF-2 α by GM-CSF. Analysis of the soluble factor content of melanoma conditioned media will serve as valuable controls for the purified exosome experiments. Further, similar lymph node *in vivo*, *ex vivo* and *in vitro* culture experiments as described previously can be employed to compare the extent of melanoma exosome induction of endothelial GM-CSF to that of soluble mediators such as IL-1 β or TNF- α .

Implications of the Hypothesis

In a hypothetical GM-CSF model scenario, induction of GM-CSF by VECs or possibly LECs in lymph nodes might be influenced by a melanoma exosome concentration gradient. For example, early in the pre-metastatic process, SCS M1 M ϕ polarization may dominate the lymph node microenvironment. Previous studies suggest that the Th1 or anti-tumor M1 lymph node microenvironment is the default state in non-tumor draining lymph nodes (3). In such a scenario, melanoma exosome induction of endothelial derived GM-CSF would increase SCS M1 M ϕ expression of HIF-1 α and production of VEGF resulting in traditional angiogenesis (**Fig. 1A**) in the context of an anti-tumor M1 immune response.

Subsequently, as tumors grow and the concentration of melanoma exosomes continues to increase in tumor draining lymph nodes, the presence of myeloid derived suppressor cells (MDSCs) in the lymph nodes might be observed. Melanoma exosomes have been shown to induce MDSCs (2). MDSCs can polarize M ϕ s toward an immunosuppressive M2 phenotype (6). Further, exposure of SCS M ϕ s to melanoma derived IL-10 (42, 43) and TGF- β (44) in the pre-metastatic tumor draining lymph node could potentially polarize SCS M ϕ s to an M2c-like phenotype (37).

IL-10 polarization of SCS M ϕ 's to an M2 phenotype (37) might contribute to tumor tolerance through M2 SCS M ϕ mediated presentation of melanoma antigens carried by exosomes. SCS M ϕ mediated immune tolerance to antigens carried by exosomes has been previously demonstrated (33). Alternatively or coincidentally, immune tolerance to melanoma exosome derived antigens might be further bolstered via interactions between LECs and DCs in lymph nodes (45).

Whether SCS M ϕ polarity influences SCS M ϕ immunoprotective barrier function remains to be determined. Conceivably, pro-tumor SCS M ϕ M2 polarization could hinder the expansion of opposing anti-tumor SCS M1 M ϕ s in the face of increased tumor growth. This could impede the capacity of SCS M1 M ϕ s to serve as an immunoprotective tumor exosome barrier. They could become overwhelmed by sheer melanoma exosome numbers.

As the SCS M2 M ϕ population becomes greater than the SCS M1 M ϕ population in the lymph node microenvironment, melanoma exosome induction of endothelial derived GM-CSF would be expected to induce more HIF-2 α expression in SCS M2 M ϕ s, possibly the M2c subclass, than HIF-1 α in SCS M1 M ϕ s. An increased ratio of sVEGFR-1 to VEGF in lymph nodes would be realized (**Fig. 1B**). This would have the effect of attenuating VEGF function in favor of increased vascular remodeling and complexity in the context of an immunosuppressive lymph node microenvironment.

Despite their opposing immune functions, indirect melanoma exosome stimulation of SCS M1 or M2 M ϕ function via endothelial derived GM-CSF in lymph nodes may induce different although complementary pro-tumor angiogenic processes. These processes may not be mutually exclusive but may occur simultaneously at times coincident with evolving tumor pathogenesis. This may help explain to some extent confounding clinical trial results evaluating GM-CSF monotherapy for melanoma.

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Figure 1.

Induction of endothelial cell derived GM-CSF by melanoma exosomes mediates different angiogenic functions in pre-metastatic lymph nodes depending on SCS macrophage polarity. Melanoma exosomes induce vascular endothelial cell (VEC) production of GM-CSF possibly through delivery of exosome shuttle GM-CSF mRNA. **(A)** GM-CSF induces HIF-1 α activity in M1 SCS M ϕ s. This results in VEGF production which stimulates VEC proliferation and angiogenesis. **(B)** Alternatively or coincidentally, GM-CSF induces hypoxia inducible factor 2 (HIF-2 α) activity in M2 SCS M ϕ s. This results in sVEGFR-1 production which attenuates VEGF activity promoting better organized and patent vasculature conducive to supporting tumor growth. The presence of factors bolded in red is supported by previous exosome studies (11, 15).