

# Cancer's smart bombs: tumor-derived exosomes target lung epithelial cells triggering pre-metastatic niche formation

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The formation of the pre-metastatic niche is a crucial step towards metastasis. In this project, Liu and colleagues demonstrate that lung primary tumor derived exosomes selectively target and activate lung alveolar epithelial type 2 cells inducing the secretion of chemokines responsible for causing lung neutrophil invasion and thus promoting the formation of the pre-metastatic niche (1).

Lung cancer metastasis is a leading cause of death in patients with lung cancer. It is now well known that metastasis is not a random process. The success of colonization of distant sites by circulating tumor cells (CTCs) depends on the local microenvironment that the CTCs encounter (2). It has been suggested that the tumor cells secrete trans acting factors that can target and critically influence the local microenvironment of specific tissues so they can be made susceptible to metastasis (3). The secondary sites that are targeted for metastatic invasion and are actively affected by primary tumor induced pro-metastatic factors are termed the pre-metastatic niche. In the last years, a considerable amount of effort has been put into understanding the mechanisms in play that cause the formation of the pre-metastatic niche loci. Among them, chronic unresolved inflammation is known to be an important driver of tumor development and metastasis (4). Many molecular components and signaling pathways are involved in the connection among regulatory immune cells, inflammation, cancer growth, and metastasis (5). Primary tumor secreted trans

acting factors have been shown to induce inflammation at secondary sites in order to support the survival and proliferation of tumor cells in the pre-metastatic niche (3). Host cell Toll-like receptors (TLRs) have been shown to be targeted by a variety of tumor secreted factors and prolonged activation of non-cancerous cell TLRs can cause chronic inflammation that promotes metastasis (6). Recent findings have shown that TLR-activating tumor-derived trans acting factors can be packed into exosomes (7), which are small membrane-bound nanoparticles that contain a large variety of proteins, RNA, DNA and lipids, and are further released by many cell types in blood and other body fluids. The tumor-derived exosomes upon release to circulation seem to act as complex signaling messengers and are taken up by specific host cells to induce a pro metastatic cellular response (7).

In this project, Liu and coworkers reveal that exosomes secreted from primary lung cancer tumor cells can activate TLR3 on alveolar epithelial type 2 cells to induce a pro metastatic chemokine response that causes neutrophil invasion in the lung (1). The authors show that TLR3 double knockout mutant mice exhibited increased resistance to metastasis in the lung after tumor inoculation as well as significant downregulation of metastatic niche related genes. They conclude that TLR3 activation plays a pivotal role in lung metastasis and TLR3 activation promotes lung metastasis by upregulating the expression of metastatic

niche related genes in the lung. Indeed, after subcutaneous tumor inoculation with Lewis lung carcinoma (LLC) or B16/F10 melanoma TLR3<sup>-/-</sup> mice showed increased survival and vastly reduced lung metastasis compared to WT mice. However, the primary tumor remained unaffected in all specimens implying that TLR3 plays a specific role in metastasis and does not affect primary tumor cancer cell survival. Interestingly the expression of metastatic niche characteristic genes including the genes encoding proinflammatory chemokines Bv8, S108, S109, extracellular matrix remodeling enzyme Mmp9 as well as fibronectin were significantly downregulated.

To investigate the types of cells that partake in the activation of the TLR3-mediated pro metastatic response, the authors compared the cellular composition of lungs from TLR3 deficient mice with wild type (WT) ones after tumor inoculation. Fluorescence-activated cell sorting analysis revealed that CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6Cint neutrophils were remarkably expanded in the pre-metastatic lung of WT mice compared to TLR3<sup>-/-</sup> mice after tumor inoculation. Interestingly the neutrophils overexpressed VEGFR1 and BV8 strongly indicating that they play an integral part in lung pro metastatic niche formation. To validate the pro metastatic effect of neutrophil invasion in the lung, the authors examined the effect of neutrophil depletion in mice after tumor inoculation and investigated the possible connection between TLR3 activation and neutrophil recruitment in the lung by comparing the neutrophil content of WT and TLR3 deficient mice. They discovered that depletion of neutrophils by administering anti Ly6G antibody halved the average number of lung metastatic nodules confirming the major role of neutrophils in lung metastasis. Furthermore, TLR3 deficient mice exhibited significantly decreased lung neutrophil accumulation and metastasis, interestingly TLR3 knock-in with recombinant adenovirus rescued the neutrophil invasion phenotype and the susceptibility to lung metastasis confirming a causal relationship between TLR3 activation and neutrophil recruitment

To discover the types of cells that are responsible for neutrophil recruitment in the lung as a result of TLR3 activation, the authors analyzed the expression of TLR3 mRNA under normal conditions and after tumor inoculation in lung stromal cells capable of producing chemokines. Neutrophils can be stimulated and further recruited to tissues, as a response to the release a specific subset of chemokines (CXCR1, CXCR2, CXCR4, and CCR2) by chemotaxis through the activation of MAPK

and NF- $\kappa$ B pathways by TLR3 (8). Quantitative rt-PCR analysis revealed that lung neutrophils did not express TLR3, indicating that neutrophil-mediated TLR3 activation is not responsible for their recruitment. However, the expression levels of TLR3 in lung epithelial cells were the highest compared to other cell types and TLR3 mRNA was significantly upregulated after tumor inoculation. Alveolar type 2 cells (AT-2) are the most prevalent type of lung epithelium consisting its 60% and contribute to lung immune defense (9). The authors examined the ability of AT-2 cells to induce TLR3-activated chemokine response when stimulated by lung tumor cells. AT-2 cells isolated from WT and TLR3-deficient mice were cultured and then inoculated with tumor cells. The expression of chemokine coding mRNAs was compared by means of a genechip experiment. It was discovered that chemokine transcripts were more than 4-fold upregulated when WT AT-2 cells were inoculated with tumor cells, while AT-2 cells derived from TLR3 deficient mice did not significantly alter the expression of chemokine mRNAs under the same conditions. These findings show that some unknown tumor derived factors may induce the production of chemokines by activating TLR3 in AT-2 cells. To examine if the exosomes are the tumor secreted trans acting factors responsible for the induction of the TLR3-mediated pro metastatic response in AT-2 cells, the authors investigated the ability of the exosomes to target AT-2 cells and to mediate lung metastasis. Exosomes transfer a variety of cargo molecules, including dsRNA that has been shown to activate TLR3. In addition, exosome transmitted cargo has been shown to activate other TLRs (TLR2, TLR4, TLR7) (10,11). To examine the ability of tumor derived exosomes to specifically target AT-2 cells in lung metastasis, WT mice were injected with fluorescently labeled exosomes isolated from lung cancer. FACS analysis showed that the labeled exosomes were quickly enriched in the lungs and taken up by AT-2 cells, confirming that exosomes actively target the abovementioned cells.

The pro metastatic effect of tumor derived exosomes was also examined. TLR3<sup>-/-</sup> and WT mice were initially injected with exosomes from lung cancer, followed by LLC cells two weeks later. It was discovered that exosomes quickly mediated the effects associated with pre-metastatic niche formation in the lung of WT mice but not in TLR3-deficient mice. Following exosome injection WT mice exhibited elevated chemokine production, overexpression of metastatic niche associated genes (BV8, s100a8, s100a9, Mmp9 and fibronectin) and neutrophil invasion, predictably

lung metastasis followed shortly after LLC cells were inoculated. By comparison, TLR3<sup>-/-</sup> mice exhibited less neutrophil accumulation in the lung, as well as significantly reduced lung metastasis. These results confirm the pro metastatic role of tumor derived exosomes in the lung. To verify that the tumor derived exosomes are responsible for activating TLR3 to induce a pro metastatic chemokine response, the authors isolated AT-2 cells from WT and TLR3 deficient mice and transfected them with exosomes isolated from LLC. In parallel, they knocked down TLR3 expression in MLE-12 cells, (derived from AT-2) before transfecting them with exosomes. It was observed that TLR3 and chemokine gene expression was upregulated after exosome stimulation on WT AT-2 and MLE-12 cells. In direct contrast, the production of chemokines was downregulated in TLR3<sup>-/-</sup> AT-2 cells or upon silencing of TLR3 expression in MLE-12 cells. These results indicate that tumor-derived exosomes are capable to induce the production of pro metastatic chemokines by activating TLR3.

The authors further examined if exosomal RNA is responsible for activating TLR3 and thus mediating AT-2 cell chemokine production. For this purpose, they transfected cultured AT-2 cells with tumor-derived exosomal RNA and observed that the production of chemokines was significantly increased, while tumor RNA had no effect. In particular, exosomal RNA but not tumor RNA induced the phosphorylation of Erk, Jnk, and p38, as well as the p65 subunit of NF-κB, members of the MAPK and NF-κB signaling pathways, respectively, known to control chemokine production. Furthermore, tumor exosomal RNA and tumor cell RNA were sequenced revealing that the composition of the two RNA groups was very different; non-coding RNAs were significantly enriched in the exosomal RNA (more than 70% of the RNAs) compared to tumor RNA. Of the non-coding RNAs, snRNAs were the most enriched RNA subtypes, where U1snRNA was 1,000-fold increase in the exosomal RNA group compared to tumor RNA. These findings suggest that exosomal RNAs are selectively packed to the exosome and imply that U1snRNA may act as TLR3 ligand.

The study of Liu and coworkers is a bold attempt to reveal the core biochemical interactions to form the pre-metastatic niche during the development of lung metastasis. The authors have successfully revealed the key players responsible for promoting metastasis by means of influencing the local microenvironment in the lung. They have also provided conclusive evidence to support

that TLR3 activation in the lung stroma is a crucial event towards the formation of the lung pre-metastatic niche. Nonetheless, it is rather unlikely that TLR3 activation alone is enough to trigger pre-metastatic niche formation in the lung. Work in the future is expected to reveal the role of distinct exosomal RNAs, which, aside from activating TLR3, may influence key components of the signaling “grid” that controls the production of chemokines downstream of TLR3.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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