M2a macrophages induce contact-dependent dispersion of carcinoma cell aggregates

Giulia Adriani¹, Jing Bai¹, Siew-Cheng Wong², Roger D. Kamm¹,³, Jean Paul Thiery¹,⁴,⁵

¹BioSystems and Micromechanics IRG, Singapore-MIT Alliance for Research and Technology, 1 CREATE Way, 138602, Singapore
²Singapore Immunology Network, Agency for Science, Technology and Research, 8A Biomedical Grove, Biopolis, 138648, Singapore
³Department of Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Ave, Cambridge, MA, 02139, USA
⁴Department of Biochemistry, National University of Singapore, MD7, 8 Medical Drive, 117597, Singapore
⁵Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, 61 Biopolis Drive, 138673, Singapore

Correspondence: Jean-Paul Thiery
E-mail: bchtjp@nus.edu.sg
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Monocytes and macrophages accumulate at the site of tumors driven by chemokines such as CCL2, monocyte chemotactic protein 1 (MCP1), vascular endothelial growth factor (VEGF), and macrophage colony stimulating factor (M-CSF). Convincing evidence suggests that tumor-associated macrophages (TAMs) promote tumor growth, epithelial-mesenchymal transition (EMT) and intravasation of blood and lymph vessels. Recently, we reported experimental findings in a microfluidic tumor model demonstrating that M2a macrophages induce contact-dependent EMT of carcinoma cell aggregates via ICAM-1 and integrin β2 interactions. These findings support the pro-metastatic activities of M2a macrophages and may help in designing novel immunotherapies for cancer patients.

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Epithelial-mesenchymal transition (EMT) is a fundamental process for embryonic development and refers to a complex cell biological program where epithelial cells lose apico-basal polarity, junctional complexes and stationary state to acquire mesenchymal traits such as migratory and invasive properties. The acquisition of a mesenchymal phenotype allows carcinoma cells to reach lymph and/or blood vessels and disseminate toward distant sites to form secondary colonies [¹,²] while a mesenchymal-epithelial transition must operate at the secondary site to allow secondary tumor growth [³,⁴].

EMT is now known as a dynamic process that includes a continuous spectrum of intermediate states between the two extreme cellular phenotypes [⁵,⁶]. Pathological EMT leads to carcinoma dissemination [²] and, thanks to the development of an EMT scoring system, it has recently been demonstrated to have the potential to become a useful tool to monitor the progression of carcinoma [⁶]. Although studies have identified matrix metalloproteinases (MMPs) [⁷], transforming growth factor-β (TGF-β) [⁸] and other cytokines in the stroma as EMT inducers, the origin of these factors is not fully understood and requires further investigation. On the other hand, accumulating observations showed that
macrophages infiltrate tumors [9] and alter the microenvironment by secreting angiogenic [10,11] and proteolytic factors [12]. These infiltrating macrophages, also referred to as tumor associated macrophages (TAMs) are plastic cells with differential cytokine production that present a dual function in terms of their interaction with carcinoma cells. Infiltrating monocytes, attracted by different chemokines such as CCL2, VEGF, and M-CSF, in fact, differentiate into a spectrum of macrophages where M1 and M2 represent the two extreme phenotypes [13]. This polarization occurs in response to different factors such as interferon-γ (IFN-γ) for the classically activated M1 macrophages and interleukin-4 (IL-4) for the alternatively activated M2 macrophages conferring immunostimulatory or immunosuppressive properties, respectively [14]. TAMs usually - but not always - have an M2-like phenotype [15,16] and their presence in the stroma has been shown to play a key role in suppressing adaptive immunity [13], triggering EMT [17-19] and promoting carcinoma cell survival [20], invasion and metastasis [21-23].

Indeed, TAMs greatly contribute to the inflammatory microenvironment common to carcinomas by secreting cytokines such as TNF-α that interact with TGF-β to induce EMT as shown in a model of colon carcinoma spheroids [24] and in a teratocarcinoma in vitro conditioned media culture assay [17]. CCL22 secreted specifically by M2 macrophages has been shown to promote EMT in hepatocellular carcinoma (HCC) in a co-culture system and an in vivo model [25]. CCL18, also secreted by M2 macrophages, instead, forms part of a feedback loop with the granulocyte-macrophage colony-stimulating factor (GM-CSF) secreted from carcinoma cells to sustain EMT, through activation of the NF-κB pathway in a co-culture system and in a humanized mouse model [19]. Specifically, NF-κB induce the expression of potent EMT inducers, including the transcription factor Snail1. Moreover, M2 macrophages promote EMT in pancreatic carcinoma through toll-like receptor 4 (TLR4)/IL-10 signaling [26].

Taken together, these findings highlight the importance of investigating the interactions between macrophages and carcinoma cells for elucidating the mechanisms underlying tumor progression. To help address this need, our group recently conducted a study on contact-dependent dissemination of lung carcinoma aggregates by M2a...
macrophages. Monocyte-derived macrophages (M0), can be differentiated in different M2 subtypes: the M2a subtype results from activation by IL-4, M2b from stimulation with LPS and culture on human IgG coated wells while M2c by activation with IL-10.

In our study, we cultured human lung adenocarcinoma (A549) cell aggregates to assess the role of distinct macrophage subtypes, namely M0, M1, M2a, M2b and M2c (Figure 1), in inducing EMT and carcinoma cell aggregate dissemination in a three-dimensional (3D) microfluidic platform. The multicellular culture system included A549 aggregates and macrophages in a 3D collagen matrix with human umbilical vein endothelial cells (HUVECs) cultured in the lateral fluidic channel to more closely mimic the in vivo microenvironment. Our platform allows real-time monitoring of interaction between carcinoma cells and macrophages, as well as precise measurements of aggregate dispersion and cell migration speed.

First, we showed by immunostaining, that M1 and M2a macrophages were expressing their respective markers (CD80 and CD209) after 36 h within the 3D collagen matrix in the presence of carcinoma aggregates and endothelial cells indicating that their phenotypes were maintained throughout the co-culture period. Then, we investigated the macrophage-assisted dispersal of carcinoma aggregates with macrophages in the same collagen gel region or in an adjacent collagen gel region that allowed for paracrine signalling but not physical contact between carcinoma cells and macrophages. We found that all macrophage subtypes promoted carcinoma aggregate dispersion 3 to 4 times greater than in the absence of macrophages. Interestingly, M2a macrophages induced greater A549 carcinoma aggregate dispersal only when they were in the same collagen gel region with the carcinoma aggregates (Figure 2).

We further investigated macrophage motility and the role of integrins in contact-dependent dissemination. By

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**Figure 2. Schematic representations of carcinoma aggregate dispersion at 0h and 36h in contact and separate condition into the microfluidic device.** First column shows the macrophage subtype and the microfluidic device (media channel in pink, media channel with HUVECs in green, 3D collagen matrix for carcinoma aggregate and macrophage interactions). Second and third columns show the schemes for the contact or the separated condition at 0h and 36h (A549 aggregates in red; M0, M1, M2b and M2c in green; M2a in blue).
time-lapse imaging, we could observe that M2a have a greater migration speed compared to all the other subtypes when cultured in the same gel region as the A549 carcinoma aggregates and when located ≤50 μm from carcinoma cells. Importantly, only M2a macrophages showed a directional migration towards the A549 aggregates to establish cell-cell contact with the carcinoma cell and promote dispersion of the carcinoma cell, indicative of an EMT occurring process. The loss of E-cadherin is a fundamental event in EMT [3] and was observed in our study. This further suggested that M2a macrophages after 36 h in close proximity with A549 aggregates were promoting EMT.

We next examined the role of integrins relevant for cell-cell adhesion. Leukocyte specific integrin α chains (CD11a, CD11b and CD11c) and the β2 chain (CD18) on macrophages were considered as well as their binding molecule ICAM-1 on carcinoma cells to confirm the significance of M2a macrophage adhesion to carcinoma cells in aggregate dispersion. We found that specifically blocking integrin CD11a, CD11b or CD18 on M2a macrophages or ICAM-1 on A549 aggregates decreased carcinoma aggregate dissociation significantly, suggesting a possible mechanism of contact-dependent dissemination via ICAM-1 and integrin β2 interactions.

In summary, our findings give new insights into how macrophages of polarized subtypes and carcinoma cells interact in the tumor microenvironment. This microfluidic 3D culture platform opens the way for further investigations into the possible mechanisms for carcinoma-macrophage signalling in EMT. Moreover, our current research focuses on co-culturing different immune cells in our tumour model to aid in testing immunotherapeutic approaches and/or screening of anti-metastatic drugs in a more complex in vivo tumour-like microenvironment.

Conflicting interests

The authors have declared that no conflict of interests exist.

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