Primary tumor hypoxia recruits CD11b+/Ly6Cmed/Ly6G+ immune suppressor cells and compromises NK cell cytotoxicity in the pre-metastatic niche

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Abstract:

Hypoxia within a tumor acts as a strong selective pressure that promotes angiogenesis, invasion and metastatic spread. In this study, we used immune competent bone marrow chimeric mice and syngeneic orthotopic mammary cancer models to demonstrate that hypoxia in the primary tumor promotes pre-metastatic niche formation in secondary organs. Injection of mice with cell-free conditioned medium derived from hypoxic mammary tumor cells resulted in increased bone marrow-derived cell infiltration into the lung in the absence of a primary tumor and led to increased metastatic burden in mammary and melanoma experimental metastasis models. By characterizing the composition of infiltrating bone marrow-derived cells, we identified CD11b+/Ly6Cmed/Ly6G+ myeloid and CD3-/NK1.1+ immune cell lineages as key constituents of the pre-metastatic niche. Furthermore, the cytotoxicity of NK cells was significantly decreased, resulting in a reduced anti-tumor response that allowed metastasis formation in secondary organs to a similar extent as ablation of NK cells. In contrast, metastatic burden was decreased when active NK cells were present in pre-metastatic lungs. Together, our findings suggest that primary tumor hypoxia provides cytokines and growth factors capable of creating a pre-metastatic niche through recruitment of CD11b+/Ly6Cmed/Ly6G+ myeloid cells and a reduction in the cytotoxic effector functions of NK cell populations.
**Introduction:**

Hypoxia is a common feature and poor prognostic marker in several solid cancers (1). Cells respond to hypoxia through stabilization of the Hypoxia-inducible factor (Hif) transcription factor, resulting in the expression of genes involved in angiogenesis, invasion and metastasis (1).

Hypoxia controls the composition of the tumor microenvironment, and the invasive and metastatic capacity of various cancers (2). Factors capable of inducing bone marrow-derived cell (BMDC) mobilization and recruitment to the tumor microenvironment include Hif targets VEGF, angiopoietin-1, PIGF, PDGF-B and SDF1α (3). Emerging evidence suggests primary tumor hypoxia and BMDCs drive pre-metastatic niche development in distant tissues, making them permissive for metastatic spread (4-5).

Pre-metastatic niche formation was first attributed to tumor-derived VEGF and PIGF-induced secretion of fibronectin from fibroblasts, promoting adhesion of VEGFR1+ hematopoietic progenitor cells (6). Additional studies reported alternate mechanisms for pre-metastatic niche formation involving TNFα, TGFβ, lysyl-oxidase (LOX), MMP2, MMP9, CXCR4 and SDF1α among others (7). Most of these are direct or indirect Hif targets and generally important in the metastatic process (1). While secretion of these factors by the primary tumor increases BMDCs in pre-metastatic organs, in depth characterization of the responsive BMDC lineages is lacking.

Here we report that conditioned medium containing monocyte chemotactic protein-1 (MCP-1/CCL2), derived from hypoxic mammary tumor cells, can induce pre-metastatic niche formation in the lungs of immune competent animals. Furthermore, we define lung-infiltrating, bone marrow-derived CD11b+/Ly6Cmed/Ly6G+ myeloid cells and NK cells with reduced anti-tumour responses, which form a pre-metastatic niche conducive to increased metastatic burden independent of tumor type.
Materials and Methods:

Mice. Female C57Bl/6 mice were used at 8-14 weeks and purchased from the Walter and Eliza Hall Institute (Melbourne). C57Bl/6 NZeg-enhanced GFP (eGFP) mice were used as bone marrow-donors for chimeras generated as previously described (6, 8). C57Bl/6 Rag2-/- c.γ-/- mice were bred and maintained at the Peter MacCallum Cancer Centre. All animal procedures were approved by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee.

Cell lines and conditioned media assays. The EO771 and PyMT mammary carcinoma and B16F10 melanoma cell lines were maintained as previously described (9-10), and infected with the pMSCV-Cherry/Luciferase lentiviral vector for sorting by flow cytometry. Conditioned media was generated by filtering serum-free, phenol red-free low glucose Dulbecco’s Modified Eagles Medium (Invitrogen) cultured on cells for 10 h under normoxic (20% O2) or hypoxic conditions (2% O2) (6). Conditioned media (300 μl) was injected intraperitoneally daily for 7 days and lungs harvested the following day. For experimental metastasis models, mice were injected with 2 x 10^5 tumor cells intravenously on day 7, as detailed in supplementary methods.

Cytokine/angiogenesis arrays. Secreted factors in conditioned media were detected using Proteome Profiler array kits (R&D Systems), according to the manufacturers’ instructions.

Flow cytometry. Flow cytometry was performed on a single cell suspension of whole lung tissue (10). Antibody details listed in supplementary methods.

51Cr release cytotoxicity assay. CD3^-NK1.1+ cells were FACS sorted from whole lung as above. 51Cr-labeled YAC-1 target cells were used in a standard 4 h NK cell cytotoxicity assay (10).

Immunofluorescence. eGFP visualization and immunofluorescence was performed on OCT inflated lungs. Sections were stained with CD11b (BD Pharmingen) and images taken on a BX-51 microscope (Olympus).

Statistical analyses. Results are expressed as mean ± SEM and analyzed by two-tailed Mann-Whitney non-parametric t-tests, p values <0.05 were considered significant (***p<0.0001, **p<0.01 and *p<0.05).
Results and Discussion:

Tumor hypoxia causes BMDC accumulation in lungs

To examine the impact of factors produced by hypoxic tumor cells on BMDC recruitment to certain organs, eGFP+ bone marrow chimeric mice were injected with normoxic (20% O2) or hypoxic (2% O2) cell-free conditioned medium (NCM and HCM, respectively) derived from PyMT-WT or Siah2−/− mammary tumor cell lines. Siah2−/− cells lack a functional hypoxic response due to failure to stabilize Hif-1α under hypoxic conditions (9). HCM-treated mice showed a significant increase in eGFP+ BMDCs in lungs, compared to mice injected with either NCM, Siah2−/− NCM or Siah2−/− HCM (Figure 1A). Clusters of eGFP+/CD11b+ cells were also more frequently observed at terminal bronchioles of the lung in WT HCM-treated mice (Figure 1B). Therefore, factors secreted from hypoxic mammary tumor cells alone, increase the accumulation of CD11b+ BMDCs in the lung.

A strong stabilization of Hif-1α protein was observed in both PyMT-WT and EO771 cells after 2 and 10 h hypoxia exposure compared to normoxia (Supplementary Figure 1A). In contrast, Hif-2α protein was only marginally stabilized under hypoxia (Supplementary Figure 1A). This suggests that formation of the pre-metastatic niche is driven by Hif-1α signaling in hypoxic tumor cells, although a contribution from exclusively Hif-2α-regulated genes cannot be excluded (11).

Factors secreted from hypoxic tumor cells promote metastasis formation

To determine whether BMDC recruitment creates a pre-metastatic niche supportive of tumor cell colonization and metastatic outgrowth, we injected NCM or HCM derived from PyMT-WT and EO771 mammary tumor cells, followed immediately by intravenous injection of Cherry-labeled PyMT-WT or EO771 tumor cells (Supplementary Figure 1B). Naïve mice intravenously injected with Cherry-labeled tumor cells alone (IV only group), without exposure to conditioned medium, served as positive controls. HCM treatment increased metastatic colonization by PyMT-WT or EO771 Cherry-positive tumor cells compared to NCM (Figure 1C & D). These data show that pre-treatment with HCM supports the colonization and outgrowth of a greater number of metastatic tumor cells in pre-conditioned lungs by modulating the microenvironment of distant tissues.

We next defined whether the pre-metastatic lung environment created by mammary tumor cell-derived HCM also supported metastasis formation of other tumor types. Mice pre-treated with NCM or HCM from PyMT-WT tumor cells were intravenously injected with B16F10 melanoma cells. We observed a significant increase in metastatic foci in lungs of
HCM compared to NCM-treated mice (Figure 2A; Supplementary Figure 2A). Hence, HCM derived from mammary tumor cells modifies lungs to be generally receptive to circulating tumor cells, suggestive of a pre-metastatic niche with reduced anti-tumor responses.

To identify factors contributing to pre-metastatic niche formation, proteins secreted from hypoxic PyMT-WT and EO771 cells were assessed. HCM contained increased MCP-1 (CCL2), G-CSF, TNF-α, VEGF, TIMP-1 and MMP-9 (Figure 2B & C; Supplementary Figure 2B). Several of these factors have been separately reported in pre-metastatic niche formation (highlighted in Supplementary Figure 2B) (7, 12-13), and thus hypoxia within the primary tumor may be the unifying process that drives pre-metastatic niche formation during tumorigenesis. MCP-1 is a member of the CC chemokine β-subfamily known to regulate the recruitment of inflammatory cells into tissues during inflammation and cancer (14), and is increased in both PyMT-WT and EO771 HCM. Neutralization of MCP-1 in PyMT-WT HCM using a MCP-1 antibody, decreased metastatic burden compared to HCM treatment alone (Figure 2D). In contrast, MCP-1 neutralization in NCM did not alter metastatic burden compared to NCM treatment alone (Figure 2D). Thus increased metastatic burden observed after HCM treatment (Figure 1C & D) can, in part, be attributed to increased MCP-1 present in hypoxic tumor cell conditioned medium.

**Recruitment of CD11b⁺/Ly6Cmed/Ly6G⁺ BMDCs to the pre-metastatic niche**

We hypothesized that the pro-metastatic effects of HCM-treatment would be determined by the type and function of immune cells present. CD11b⁺/Gr-1⁺ myeloid cells have been previously described in the pre-metastatic niche (5, 7, 12-13, 15). This heterogeneous group of cells includes precursors of macrophages, granulocytes, dendritic cells and myeloid cells at various stages of differentiation (16). Examination of bone marrow-derived myeloid cells for Ly6G⁺ and Ly6C⁺ subtypes (both recognized by the Gr-1 antibody) revealed that only CD11b⁺/Ly6C⁺ cells were significantly increased in the lungs of HCM-treated mice (Figure 3A). Two subpopulations of CD11b⁺/Ly6C⁺ cells with different levels of Ly6C expression (denoted Ly6Cmed and Ly6Chigh) were identified (Figure 3B). Only the CD11b⁺/Ly6Cmed cells (Figure 3B middle panel), but not CD11b⁺/Ly6Chigh cells (Figure 3B right panel), were significantly increased in HCM-treated mice. Ly6G was co-expressed on more than 75% of CD11b⁺/Ly6Cmed cells in both groups compared to 30% of CD11b⁺/Ly6Chigh cells (Figure 3C). This defines two distinct subsets of Ly6C⁺ myeloid cells, CD11b⁺/Ly6Chigh/Ly6G⁻ and CD11b⁺/Ly6Cmed/Ly6G⁺, with only the latter increased in pre-metastatic organs after HCM treatment.
The CD11b⁺/Ly6C<sup>med</sup>/Ly6G<sup>+</sup> myeloid cells represent the granulocytic subset of a heterogeneous class of myeloid cells termed myeloid-derived suppressor cells (MDSCs) (16). The monocytic fraction (CD11b⁺/Ly6C<sup>high</sup>/Ly6G⁻) suppresses CD8<sup>+</sup> T cell and natural killer (NK) cell function, but the role of the granulocytic fraction is still unclear (16). It is described however, that the suppressive function of MDSCs is regulated by hypoxia, with Hif-1α considered vital in controlling their differentiation into immunosuppressive macrophages (16).

We next determined if CD11b⁺/Ly6C<sup>med</sup>/Ly6G<sup>+</sup> myeloid cells in the pre-metastatic niche controlled metastasis formation. As neutralization of MCP-1 in HCM decreased metastatic tumor burden (Figure 2D), we assessed if the myeloid cell composition of the pre-metastatic niche was also altered after MCP-1 neutralization. CD11b⁺/Ly6C<sup>med</sup>/Ly6G<sup>+</sup> but not CD11b⁺/Ly6C<sup>high</sup>/Ly6G⁻ cells were reduced when MCP-1 was neutralized in HCM but not NCM-treated mice (Supplementary Figure 3A & B). Thus, accumulation of granulocytic CD11b⁺/Ly6C<sup>med</sup>/Ly6G<sup>+</sup> myeloid cells, driven by hypoxic tumor cell-derived MCP-1, regulates the pro-metastatic properties of the pre-metastatic niche.

**NK cells in the pre-metastatic niche control metastasis formation**

We next investigated other BMDC populations in the pre-metastatic niche. While the abundance of CD8<sup>+</sup> and CD4<sup>+</sup> T cells, dendritic cells (CD11c<sup>+</sup>/MHC class II<sup>+</sup>) and macrophages (CD11b<sup>+</sup>/MHC class II<sup>+</sup>/F480<sup>+</sup>) was unchanged (Supplementary Figure 4A), CD<sup>+</sup>/NK1.1<sup>+</sup> NK cells were enriched in lungs of HCM-treated mice (Figure 3D). Characterization of these NK cells showed no difference in expression of the early activation marker CD69 (Supplementary Figure 4B), whereas expression of the differentiation markers CD11b and CD27 (17) was significantly altered in the lungs of HCM-treated mice (Figure 4A left panel). The transition from CD11b<sup>low</sup>/CD27<sup>low</sup> to CD11b<sup>high</sup>/CD27<sup>low</sup> defines immature and mature NK cells respectively, with a higher abundance of terminally differentiated mature CD11b<sup>high</sup>/CD27<sup>low</sup> NK cells found in the lung (17). We observed mature NK cells in lungs of untreated and NCM-treated mice (Figure 4A right panel), whereas HCM treatment resulted in reduced NK cell maturity (Figure 4A left panel), suggesting that HCM-derived factors hinder the differentiation of NK cells in the pre-metastatic niche. Comparing the cytotoxic effector capabilities of NK cells, HCM treatment resulted in lung NK cells with a markedly reduced capacity to kill YAC-1 target tumor cells (Figure 4B). Therefore, while HCM-treated mice demonstrated increased NK cell numbers in the pre-metastatic niche, these NK cells were overall less mature and poorly cytotoxic.
As effector lymphocytes of the innate immune system, NK cells are important in regulating metastasis, but have decreased cytotoxicity in both cancer patients and tumor-bearing mice (18). We hypothesized that the increase in metastasis observed in HCM-treated mice could be explained by the decreased ability of NK cells to eliminate incoming tumor cells, and used two models of in vivo NK cell depletion to investigate this.

Rag2<sup>−/−</sup>c.γ<sup>−/−</sup> mice which lack B, T and NK cells, were treated with NCM or HCM before injection with Cherry-positive PyMT-WT tumor cells. In contrast to immune competent models (Figure 1C & D), we found no difference in metastatic burden after NCM or HCM treatment (Figure 4C). To investigate the effect of NK cells on metastasis formation in immune competent mice, we specifically depleted NK cells with anti-asialoGM1 (Supplementary Figure 4C). There was no significant change in tumor cells in the lungs of anti-asialoGM1/HCM compared to isotype/HCM-treated mice (Figure 4D). In contrast, ablation of NK cells in anti-asialoGM1/NCM-treated mice resulted in a significant increase in tumor cells compared to isotype/NCM-treated mice (Figure 4D), suggesting that the lower metastatic burden in NCM-treated mice is mediated by anti-tumor activities of NK cells. Hence, molecules released by hypoxic primary tumor cells can reduce NK cell cytotoxic capacities, thus disabling the major innate anti-tumor response and creating a permissive niche for metastasis.

Our data demonstrates that hypoxic mammary tumor cells secrete a variety of cytokines and growth factors, including MCP-1, to create a pre-metastatic niche populated by a specific subset of myeloid cells (CD11b<sup>+</sup>/Ly6C<sub>med</sub>/Ly6G<sup>+</sup>). These findings suggest that the reduced anti-tumor activity of NK cells in the pre-metastatic niche may be attributed to the concomitant increase of CD11b<sup>+</sup>/Ly6C<sub>med</sub>/Ly6G<sup>+</sup> myeloid cells. In support of this, myeloid cell accumulation has been inversely correlated with suppression of NK cell function in a tumor-specific and contact-dependent manner in murine tumor models (18-20). The exact nature of this relationship in the pre-metastatic niche needs to be addressed in future studies. Our work uncovered, for the first time, that a population of NK cells with reduced cytotoxicity accumulate in the pre-metastatic niche, creating a microenvironment supporting metastatic growth of disseminated tumor cells.
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References:
Figure legends

Figure 1. HCM induces eGFP+/CD11b+ BMDC infiltration into lungs.
A) Percentage eGFP-positive BMDCs in the lungs of eGFP-bone marrow chimeric mice injected with PyMT-WT or Siah2−/− NCM/HCM (n=5/group). B) Lung sections from (A) co-stained with CD11b and DAPI, identify clusters of eGFP+/CD11b+ cells (indicated by arrowhead). Five images per section (4-5 mice/group) were counted and summarized graphically. C/D) Cherry-positive tumor cell burden in lungs analysed by flow cytometry in PyMT-WT (C) and EO771 (D) models at 2 and 4 weeks respectively post injection (n=3 UT (untreated)/IV only groups; n=5 NCM/HCM). Mean percentage ± SEM.

Figure 2. The hypoxia-driven pre-metastatic niche is pro-metastatic.
A) B16F10 metastatic foci in lungs of PyMT-WT NCM or HCM-treated mice counted by dissecting microscope 2 weeks post tumor cell injection (indicated by arrowheads in representative images) (n=10/group). Mean ± SEM. B/C) Cytokine (B) and angiogenesis (C) arrays were incubated with PyMT-WT or EO771 NCM and HCM. Factors increased (red boxes) or decreased (blue boxes) in HCM are listed in Supplementary Figure 2B. D) Flow cytometry analysis of lungs 4 weeks post Cherry-positive tumor cell injection and treatment with PyMT-WT NCM/HCM alone, or neutralized with MCP-1 antibody (n=4-5). Mean percentage ± SEM.

Figure 3. CD11b+/Ly6Cmed/Ly6G+ and CD3+/NK1.1+ cells are increased in the lung under hypoxia.
A) Flow cytometry analysis of Gr-1, Ly6G and Ly6C CD11b cell populations within eGFP+ BMDC lung infiltrate in PyMT-WT NCM and HCM treated mice (n=10/group). B) Representative flow cytometric plots of distinct CD11b+/Ly6Cmed (grey line) and CD11b+/Ly6Chigh (black line) subpopulations. Quantification of CD11b+/Ly6Cmed (middle panel) and CD11b+/Ly6Chigh (right panel) cells in lungs of UT (n=5), PyMT-WT NCM and HCM-treated mice (n=11/group). C) Ly6G positivity in CD11b+/Ly6Cmed and CD11b+/Ly6Chigh subpopulations. D) Representative flow cytometric plots for CD3+/NK1.1+ cells (circled) and total percentage in lungs from UT (n=4), PyMT-WT NCM and HCM-treated mice (n=11/group). Mean percentage ± SEM.

Figure 4. NK cell cytotoxicity is reduced in HCM-induced pre-metastatic niche.
A) Flow cytometry analysis of percent CD3+/NK1.1+ cells that are CD11blow/CD27low (left panel) or CD11bhigh/CD27low (right panel) from lungs of NCM and HCM-treated mice (UT
n=9/group; NCM/HCM n=11/group). Mean percentage ± SEM. **B** $^{51}$Cr release cytotoxicity assay for percent lysis of target YAC-1 cells by effector NK cells at indicated effector to target ratios (n=3 independent experiments in duplicate). **C/D** Flow cytometry analysis for percent Cherry-positive PyMT-WT tumor cells in lungs 4 weeks post injection in (C) Rag2$^{-/-}$ c.γ$^{-/-}$ (n=6/group) or mice treated with (D) asialoGM1 antibody (n=6/group) or isotype (n=3/group), and pre-treated with PyMT-WT NCM or HCM. Mean percentage ± SEM.
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