Metabolic origins of spatial organization in the tumor microenvironment

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The genetic and phenotypic diversity of cells within tumors is a major obstacle for cancer treatment. Because of the stochastic nature of genetic alterations, this intratumoral heterogeneity is often viewed as chaotic. Here we show that the altered metabolism of cancer cells creates predictable gradients of extracellular metabolites that orchestrate the phenotypic diversity of cells in the tumor microenvironment. Combining experiments and mathematical modeling, we show that metabolites consumed and secreted within the tumor microenvironment induce tumor-associated macrophages (TAMs) to differentiate into distinct subpopulations according to local levels of ischemia and their position relative to the vasculature. TAMs integrate levels of hypoxia and lactate into progressive activation of MAPK signaling that can induce tissue repair and revascularization and treat intratumoral heterogeneity. We propose that gradients of extracellular metabolites act as tumor morphogens that impose order within the microenvironment, much like signaling molecules convey positional information to organize embryonic tissues. Unearthing embryology-like processes in tumors may allow us to control organ-like tumor features such as tissue repair and revascularization and treat intratumoral heterogeneity.

Tumors display a large degree of genetic and phenotypic heterogeneity that hampers diagnosis and treatment (1–8). However, tumors are capable of local organization and respond collectively to signals from their microenvironment (9, 10). Examples include the formation of multicellular structures such as blood vessels (11, 12), coordinated collective invasion (13), noncell autonomous paracrine effects (14), and “division of labor” between different tumor cells (15). How can multicellular organization emerge within heterogeneous and genetically diverse tumors? Here we reveal that gradients of metabolites, formed by the altered metabolism of cancer cells (16–18) and accentuated by aberrant vascularization (11, 19–21), lead to predictable phenotypic diversity in the tumor microenvironment. This drives temporal and spatial coordination of multiple cell types, including tumor-associated macrophages (TAMs) that are known to respond to extracellular metabolite levels (21–24). We propose that the topology of the vasculature leads to local differences in metabolite concentrations that provide spatial information that can modulate the phenotypes of different cells in the tumor microenvironment.

Results
Extracellular Metabolites Form Gradients That Convey Positional Information in Tumors. To measure intratumoral cellular heterogeneity, while preserving information regarding tissue microarchitecture, we developed an image cytometry approach that combines multiscale microscopy and image processing to extract single-cell data, including spatial features such as distance to the vasculature, for thousands of cells in tumor tissue cryosections (Fig. 1A and Fig. S1 A–D). In a mouse model of breast tumor [mouse mammary tumor virus–polyoma virus middle T-antigen (MMTV-PyMT)] we found that gradients of hypoxia [labeled with pimonidazole (PMO)] closely mirrored the topology of the vasculature and, consistent with previous reports (12, 19–21), saturated at ~100 μm from the closest blood vessel (Fig. 1B and C).

We asked whether these gradients of metabolites could impose patterned phenotypic changes in cells experiencing different local conditions. We focused on TAMs, a major prometastatic stromal infiltrate (25–27) whose phenotypic state and viability can be altered by metabolic stimuli (21–23). We measured levels of selected markers associated with different TAM states and investigated their relationship to local hypoxia levels and distance to the nearest vessel. Whereas some macrophage markers were expressed homogeneously (Fig. S1E), the TAM markers arginase 1 (ARG1) and mannose receptor, C type 1 (MRC1) were expressed in distinct and spatially restricted subpopulations (Fig. 1D). TAMs located in well-nourished regions, such as cortical and perivascular areas, expressed MRC1 whereas ARG1 was expressed in areas of hypoxia.

Significance
Cancers appear as disordered mixtures of different cells, which is partly why they are hard to treat. We show here that despite this chaos, tumors show local organization that emerges from cellular processes common to most cancers: the altered metabolism of cancer cells and the interactions with stromal cells in the tumor microenvironment. With a multidisciplinary approach combining experiments and computer simulations we revealed that the metabolic activity of cancer cells produces gradients of nutrients and metabolic waste products that act as signals that cells use to know their position with respect to blood vessels. This positional information orchestrates a modular organization of tumor and stromal cells that resembles embryonic organization, which we could exploit as a therapeutic target.

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macrophages within hypoxic regions, far from the vasculature, showed high ARG1 protein levels (Fig. 1 D and E). The spatial segregation between ARG1hi and MRC1lo TAMs was somewhat surprising because these markers are often coexpressed with antifibrotic macrophages that respond to type 2 helper T-cell (Th2) signals and have been proposed to share features with TAMs (26–29). We confirmed the correlation between hypoxia and the location of ARG1hi TAMs in a mouse model of pancreatic neuroendocrine tumors [rat insulin promoter 1–T-antigen (RIP1-TAg2)] (Fig. S1F), indicating that spatial patterning of TAM phenotypes is a common feature of the tumor microenvironment.

Gradients of Oxygen and Lactate Orchestrate Spatial Patterns of Cell Phenotypes. Our in vivo observations suggest a model where extracellular metabolites specify different TAM subpopulations across concentration gradients, similar to how morphogen gradients specify stripes of gene expression during embryonic development (30–32). We then sought to investigate whether metabolite gradients were sufficient to produce gene expression patterns in TAMs. Traditional in vitro systems are spatially homogeneous without major changes in metabolite distribution. To overcome this, we developed an in vitro microphysiological system named the metabolic microenvironment chamber (MEMIC) (Fig. 2A and B). MEMIC allows gradients of ischemia to emerge spontaneously as a result of cellular activities such as nutrient consumption and secretion of waste products. As a proof of principle, we used MEMIC to generate and visualize oxygen gradients in the hypoxic response of C6 glioma cells engineered to express GFP under an HIF1α-responsive element (C6-GFP-HRE) (21) (Fig. 2B).

To assess whether gradients of metabolites can pattern macrophages in the MEMIC, we cultured bone marrow-derived macrophages (hereafter referred to simply as macrophages) (SI Materials and Methods) with C6-GFP-HRE cells. After 24 h, macrophages translated into discrete stripes of gene expression patterns with clear boundaries (Fig. 2F and Fig. S2C). To identify which metabolites are setting these phenotypic boundaries, we focused on the ischemic ARG1hi/MRC1lo macrophage subpopulation. First, we wanted to separate the effect of hypoxia from other ischemic conditions such as nutrient deprivation. To do this, we cultured macrophages within a modified MEMIC where the top cover was made permeable to oxygen but not to soluble metabolites. In this scenario, ARG1 expression emerges as a stripe between well-nurtured macrophages and the region where ischemic levels become lethal (Fig. 2F). Together, these results demonstrate that MEMIC experiments suggest that extracellular metabolite gradients play a key role in the phenotypic diversity of TAMs.

Gradual changes in metabolite concentration are somehow translated into discrete stripes of gene expression patterns with clear boundaries (Fig. 2F and Fig. S2C). To test whether hypoxia was sufficient to induce ARG1, we cultured macrophages in regular culture plates within a hypoxic chamber (1% oxygen) or under normal cell culture conditions (21% oxygen). Surprisingly, we found no evidence for the synergistic role of hypoxia and lactate. First, the treatment of sparse macrophage populations with a combination of hypoxia and culture media previously conditioned by dense macrophage populations (cultured under hypoxia) increased ARG1 levels (Fig. 2F). To determine the molecular weight of the secreted signal, we fractionated the conditioned media (3-kDa cutoff columns). This revealed that the low, but not the high, molecular weight fraction of the conditioned media increased ARG1 levels in sparse macrophage cultures, indicating that the sensed factor was a small molecule (Fig. 2F). We therefore screened for different small molecules that could synergize with hypoxia to increase ARG1 levels (Table S1). We found
that the most potent inducer was lactate (Fig. S3B). Hypoxic macrophages secreted lactate, which accumulated in the cell culture media proportionally to the density of macrophages (Fig. 2F). When we treated normoxic and hypoxic macrophages with a wide range of lactate doses, we saw a concomitant increase of ARG1 protein levels in hypoxic macrophages but not in normoxic ones, showing that lactate and hypoxia act as synergistic cues to induce ARG1 expression (Fig. 2K). This effect is consistent with recent evidence showing a similar effect of lactate in transcription levels of Arg1 mRNA in TAMs (22). However, in our experiments, lactate alone was not sufficient to trigger a maximal Arg1 response at the protein level (Fig. 2K).

Lactic acid had similar effects to those of lactate but at high concentrations it also affected macrophage viability due to media acidification (21) (Fig. S3 C–E). Overall, our results showed that a combination of low oxygen and high lactate was necessary and sufficient to trigger the expression of ARG1 in macrophages. These data suggest that opposing gradients of lactate and oxygen, which are ubiquitous in solid tumors (19–21), create predictable phenotypic patterns among TAMs. Because these gradients follow the topology of the tumor vascular system (Fig. 1), it is possible that they convey positional information about the distance to blood vessels.

**Positional Information Is Interpreted via MAPK/ERK Signaling.** We then investigated how macrophages sensed the combination of low oxygen and high lactate and integrated these signals into phenotypic responses. We used RNA sequencing (RNA-seq) to identify transcriptional differences between macrophages treated with different levels of oxygen and/or high lactate (Fig. 3A and S3 A–C). We first observed that transcriptional changes in macrophages treated by oxygen/lactate did not display a consistent change in gene expression of markers commonly used to characterize macrophages as pro- or antiinflammatory (26–29) in a consistent manner (Fig. S4C). The effect of metabolites, however, did induce features of the Toll-like receptor 4 (TLR4)-induced...
macrophage response to LPS that signals via the MAPK pathway (Fig. S4 C and D), such as Arg1 and Nos2 coexpression (34). Reinforcing this observation, gene set enrichment analysis (GSEA) of RNA-seq data revealed that macrophages cultured in low oxygen and high lactate showed an enriched signature of KRAS/MAPK signaling activation (Fig. 3A and Fig. S4F). Pharmacological perturbations further supported a role for the MAPK pathway by showing that LPS increased ARG1 levels in hypoxic macrophages specifically through this pathway (Fig. S4 E—H).

If the MAPK pathway is interpreting positional information brought by oxygen and lactate gradients, it should (i) be active in a gradual manner and (ii) be required for the phenotypic switch we observed in macrophages (35, 36). First, we observed a strong correlation between phospho-ERK1/2 levels and the distance from the normoxic region (Fig. 3B) whereas total MAPK (ERK1/2) levels remained constant (Fig. S4I). Inhibition of MEK, a key member of this pathway, completely abrogated ARG1 patterning (Fig. 3C). Similar results were obtained by inhibiting the upstream MAPK component c-Raf with the Food and Drug Administration (FDA)-approved inhibitor Sorafenib as well as with the more selective GW5074 (Fig. S4K). Altogether our data show that, by sensing lactate and oxygen levels, TAMs can determine their position with respect to the vasculature (or to the slit in the MEMIC). This positional information required MAPK signaling and led to a predictable emergence of spatial phenotypic diversity in macrophages.

Macrophages Relay Positional Information and Organize Morphogenetic Changes in Neighboring Cells. During embryogenesis, patterned cells can elaborate and refine developmental programs by relaying their positional information via the secretion of cell signals that are sensed by adjacent cells (30–32). We thus hypothesized that macrophages, conditioned by metabolic cues, can relay their positional information to neighboring cells (Fig. 4A). We thus compared cytokine secretion profiles of macrophages treated with hypoxia and/or high levels of lactate. The combination of hypoxia and lactate increased secretion of the proangiogenic cytokine VEGFA above all other 11 screened cytokines (Fig. 4B). Quantification of secreted VEGFA, using a bead-based ELISA (Fig. 4C), and of Vegfa mRNA levels, using quantitative PCR (qPCR) (Fig. S4D), showed that hypoxia increased VEGFA secretion and that this effect was boosted by lactate, indicating that hypoxia and lactate regulate macrophage cytokine production in a synergetic manner (Fig. S4F).

To test whether these cytokines were functionally relevant, we cocultured GFP-expressing macrophages with mCherry-labeled SVEC4-10 endothelial (SVEC) cells embedded in a matrigel/collagen I matrix layer (Fig. S5B). Under normal conditions, these cocultures did not result in any evidence of vasculogenesis. In contrast, macrophages previously cultured under low oxygen and high lactate levels were able to induce tube-like morphogenesis in SVEC cells (Fig. S5B and Movie S1). Control experiments using hypoxia and lactate in the absence of macrophages or in the presence of macrophages treated with MAPK or VEGF inhibitors showed no vascular morphogenesis, indicating that VEGFA, secreted by ischemic macrophages, was required for the response of endothelial cells (Fig. S5C). These data show that macrophages were able to relay information about microenvironmental conditions to endothelial cells and to trigger a modular and noncell autonomous process of tube-like morphogenesis in response to oxygen and lactate levels.

Positional Information Optimizes Angiogenesis and Leads to Faster-Growing Tumors. Lactate and hypoxia increase with the distance from the vasculature, suggesting that VEGFA levels should also increase within ischemic regions. Using an agent-based model that we developed to study this type of question (21), we predicted that VEGFA levels should indeed increase in ischemic regions (Fig. 4D). Consistent with this, analysis of tumor sections in the MMTV-PyMT model revealed that hypoxic TAMs expressed higher VEGFA levels than TAMs located in other tumor regions (Fig. 4E). We also observed that within hypoxic tumor regions, VEGFA-expressing TAMs were associated with small groups of CD31† endothelial cells that have not yet formed into vessels (Fig. 4F). These endothelial cells stained positive for the nascent capillary marker nestin (NES) (Fig. S5A). A similar scenario has been reported during nerve regeneration where hypoxia triggers VEGFA secretion by resident macrophages that then recruit endothelial cells required for revascularization and repair (23, 37). Our data suggest that ischemic TAM subpopulations secrete VEGFA to induce endothelial cells to initiate the revascularization of nutrient-deprived tumor regions, which in turn would promote tumor growth (Fig. 4G). In fact, the metabolism of TAMs has been recently shown to control tumor blood vessel morphogenesis and metastasis (24). Is there any advantage for the tumor to maintain a spatially regulated vascularization mechanism? How does it compare, for example, to constitutive secretion of VEGFA that would promote angiogenesis throughout the tumor? With the help of mathematical modeling, we first simulated the growth of tumors with a responsive angiogenic strategy, where the secretion of proangiogenic factors (e.g., VEGFA) is modulated by extracellular metabolites, which we compared with simulated tumors that used a constitutive proangiogenic strategy. We assumed that (i) the benefits of vascularization (i.e., nutrients, oxygen) have diminishing returns on cell growth (i.e., excessive nutrients no longer increase cell growth rate) and (ii) the formation of new vessels is a costly process. We implemented this as an explicit cost, but it also resulted in oxygen resistance to flow in a dense vascular network (38). The analytical solution to this model revealed that the responsive strategy always led to faster tumor growth rates even though the total levels of VEGFA were higher in the constitutive model (Fig. 4 H and I and Fig. S6A). More detailed agent-based models confirmed these conclusions (Fig. S6 B—E, Movie S2, and Fig. 4 J and K; model details in Supporting Information, Mathematical Modeling). This result highlights that the spatial
Macrophages relay their positional information to endothelial cells and orchestrate efficient tube morphogenesis. (A) Our data suggest that, in a process resembling embryological organization, gradients of extracellular metabolites convey positional information that modifies TAM phenotypes. We asked whether additional signaling molecules, secreted by patterned macrophages, could relay positional information to other tumor cells. (B) Screening of 111 secreted chemokines revealed that ischemic macrophages express VEGFA. (C) Quantification of secreted chemokines confirmed this finding and the synergy between lactate and hypoxia (bars indicate SD from six biological replicates; **P < 0.01, ***P < 0.001). (D) Mathematical modeling predicted spatial patterns of VEGFA levels. (E) Quantification in a representative PyMT-MMTV tumor section showing that ischemic TAMs express higher VEGFA levels. (F) Images showing small groups of endothelial cells in hypoxic regions (yellow arrows). One of these regions was magnified to show that these endothelial cells are adjacent to VEGFA-expressing TAMs. For clarity, F, Lower shows isolated channels. (G) Our data suggest that whereas tumor growth leads to ischemic regions, the response of the stroma is to revascularize these regions, thus allowing tumor growth to resume. (H and I) Analytical solution of our theoretical model showed that a proangiogenic strategy that responds to ischemia (Res, responsive) led to faster-growing and larger tumors than homogeneous (Con, constitutive) angiogenesis. (J) Spatial patterns of predicted levels of VEGFA secretion for responsive and constitutive strategies. (I) Growth rates and cost of secreting VEGFA for the two strategies. (J and K) Simulation results from an agent-based model. (K) Growth curves of tumors using different proangiogenic strategies confirmed that the responsive strategy leads to enhanced tumor growth compared with the constitutive strategy. J, Right shows representative images of model outcomes. The responsive strategy not only allowed for faster tumor growth, but also required less total VEGFA. (K) Bars indicate SD from 10 simulations. (L) Localized tube morphogenesis emerges within the MEMIC from a triple coculture of macrophages, endothelial cells (SVECs), and tumor cells (T51, unlabeled). L, Inset shows a representative magnified region.

Fig. 4. Macrophages relay their positional information to endothelial cells and orchestrate efficient tube morphogenesis. (A) Our data suggest that, in a process resembling embryological organization, gradients of extracellular metabolites convey positional information that modifies TAM phenotypes. We asked whether additional signaling molecules, secreted by patterned macrophages, could relay positional information to other tumor cells. (B) Screening of 111 secreted chemokines revealed that ischemic macrophages express VEGFA. (C) Quantification of secreted chemokines confirmed this finding and the synergy between lactate and hypoxia (bars indicate SD from six biological replicates; **P < 0.01, ***P < 0.001). (D) Mathematical modeling predicted spatial patterns of VEGFA levels. (E) Quantification in a representative PyMT-MMTV tumor section showing that ischemic TAMs express higher VEGFA levels. (F) Images showing small groups of endothelial cells in hypoxic regions (yellow arrows). One of these regions was magnified to show that these endothelial cells are adjacent to VEGFA-expressing TAMs. For clarity, F, Lower shows isolated channels. (G) Our data suggest that whereas tumor growth leads to ischemic regions, the response of the stroma is to revascularize these regions, thus allowing tumor growth to resume. (H and I) Analytical solution of our theoretical model showed that a proangiogenic strategy that responds to ischemia (Res, responsive) led to faster-growing and larger tumors than homogeneous (Con, constitutive) angiogenesis. (J) Spatial patterns of predicted levels of VEGFA secretion for responsive and constitutive strategies. (I) Growth rates and cost of secreting VEGFA for the two strategies. (J and K) Simulation results from an agent-based model. (K) Growth curves of tumors using different proangiogenic strategies confirmed that the responsive strategy leads to enhanced tumor growth compared with the constitutive strategy. J, Right shows representative images of model outcomes. The responsive strategy not only allowed for faster tumor growth, but also required less total VEGFA. (K) Bars indicate SD from 10 simulations. (L) Localized tube morphogenesis emerges within the MEMIC from a triple coculture of macrophages, endothelial cells (SVECs), and tumor cells (T51, unlabeled). L, Inset shows a representative magnified region.

Discussion

Tumor progression is commonly seen as a deregulated, chaotic process, yet our data suggest that gradients of metabolites act as tumor morphogens and provide a local source of organization by inducing phenotypic patterns in macrophages. This process requires three simple conditions that are common in all solid tumors: alterations in cell metabolism, aberrant vascularization, and the presence of responsive stroma. These features lead to gradients of metabolites that orchestrate a coordinated and spatially organized angiogenic response.

It is possible that metabolite gradients also organize other cell types within the tumor microenvironment, including other immune cells (40–45) and malignant cells themselves. For example, low glutamine levels found in blood-deprived necrotic tumor cores lead to histone hypermethylation and dedifferentiation.

distribution of signals can be more relevant for the progression of the disease than its total levels, which could explain conflicting outcomes in anti-VEGFA therapies (12, 39). Our model also shows that tumors, by engaging with infiltrated macrophages, can activate a mechanism of revascularization that allows the delivery of nutrients where they are most needed without wasting resources in well-perfused tumor regions.

Our data and mathematical models suggest that proangiogenic strategies are restricted to ischemic regions, which would lead to local and responsive angiogenic strategies. To test this, we took advantage of the MEMIC system to generate regions of hypoxia in a triple coculture of PyMT-derived tumor cells, macrophages, and SVEC endothelial cells. After 3 d of culture, SVEC cells formed well-structured tubes but only within ischemic regions of the MEMIC (Fig. 4L). These data indicate that macrophages indeed orchestrate tube-like morphogenesis in regions where revascularization is most needed. More broadly, this suggests that macrophages conditioned by metabolic gradients can relay spatial information to neighboring cells, triggering functional adaptations to their local environment.
of cancer cells (46). Extracellular metabolite sensing is at the core of biological organization and is conserved from bacteria to mammals (47). Thus, we speculate that metabolites represented the primal cues for positional information, which was then coopted by signaling molecules during metazoan evolution. Understanding the processes required for multicellular self-organization in 3D may allow identification of targetable features that are independent of specific genetic mutations and thus less prone to therapeutic resistance.

Materials and Methods

Cells were cultured under standard conditions, using DMEM supplemented with 10% (vol/vol) FBS. Images were acquired using an inverted wide-field fluorescent microscope (Zeiss AxioObserver.Z1) and the images were processed in Matlab, using custom-made analysis routines that are available upon request. All animal studies were performed using protocols approved by the Animal Care Committee at Memorial Sloan Kettering Cancer Center. Details about the analytical solution to the theoretical model can be found in SI Materials and Methods.

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