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## COMPREHENSIVE IMMUNE PROFILING UNRAVELS EVOLUTION OF SPATIAL DISTRIBUTION AND IMMUNE REPERTOIRE IN TUMOR MICROENVIRONMENT FROM PRIMARY TO METASTATIC TRIPLE NEGATIVE BREAST CANCERS

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**Background** Immune checkpoint inhibitors (ICI) have successfully improved the survival in metastatic triple-negative breast cancer (mTNBC), but benefit is limited to PD-L1 positive tumors. Metastatic tumors are notorious for deficient immune cell infiltration which may limit responses to ICI in mTNBC. However, the underlying mechanisms for the weak immunogenicity of the metastatic tumor immune microenvironment (TIME) and related poor ICI responses are still not well understood. The current study was designed to investigate the evolution of the TIME between paired primary and metastatic TNBCs.

**Methods** We analyzed spatial distribution of 37 key immune regulators using the NanoString digital spatial profiling (DSP) platform. 452 regions of interests (ROIs) from 33 primary tumors (PT) and 29 metastatic tumors (MT) including 28 paired specimens, were selected based on CD45+ immune hotspots, and the protein expression levels of the key immune regulators were quantified within pan-cytokeratin (panCK) and CD45 masked regions, respectively. In parallel, we examined the clonality of tumor infiltrating B cell receptors by reconstructing the immune repertoire from bulk RNA-seq data.

**Results** Using the DSP platform, reduced immune infiltration (e.g., CD3 and CD20) in both panCK and CD45 masked regions of MT was confirmed, while CD8A and CD11c ( $p_{\text{adj}} = 2.8 \times 10^{-7}$  and  $2.1 \times 10^{-6}$ ) expression was only observed in panCK masked regions of MT compared with PT. In support of the lower CD20 counts in MT, immune repertoire analysis revealed B cell receptor (BCR) repertoire diversity (represented by Gini index) was substantially lower in MT than PT ( $p = 0.041$ ) suggesting that the ability of B cells to recognize a wide variety of tumor antigens in MT is greatly reduced in contrast to PT. Besides, we identified a significant shift in myeloid composition between PT and MT as evidenced by increased CD68 signal ( $p_{\text{adj}} = 5.8 \times 10^{-4}$ ) in CD45 masked regions of MT. Within MT, PD-L1 signal was substantially higher ( $p_{\text{adj}} = 0.030$ ) in CD45 masked regions only, while PD1 counts were lower ( $p_{\text{adj}} = 0.035$ ) in panCK masked regions. This suggests the limited responses to ICI for MT may stem from relatively low expression of activated and targetable T cell subsets in MT islands.

**Conclusions** Through comprehensive analysis of the TIME spatial organization within paired PT and MT, a significant reduction in dendritic cell/macrophage ratios (CD11c/CD68), reduced tumor localized T cell activation (CD8, PD1, PD-L1), and reduced B cell diversity (BCR clonality) are key features of the reduced immunogenicity of the metastatic TIME in TNBC.

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**Ethics Approval** Paired TNBC specimens were identified through a City of Hope IRB-approved retrospective protocol (IRB 07047) via the City of Hope (COH) Biorepository from patients with breast cancer treated at COH from 2002 to 2015. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all of the participants of this study.

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