

LYMPH NODE COLONIZATION PROMOTES DISTANT TUMOR METASTASIS THROUGH THE INDUCTION OF TUMOR-SPECIFIC IMMUNE TOLERANCE

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Background Most cancer-associated deaths result from distant organ metastasis, yet the mechanisms that promote this process remain poorly understood. For most solid tumors, colonization of lymph nodes (LNs) precedes the formation of distant organ metastases. Whether such LN metastases play a functional role in disease progression, however, has been debated for over a century. LNs are major sites of anti-tumor lymphocyte education, including in the context of immunotherapy, yet LN metastasis correlates with further disease progression.

Methods Through *in vivo* passaging of a non-metastatic syngeneic melanoma, we generated 300 unique cell lines exhibiting varying LN metastatic proclivity. Mice with parental or LN metastatic tumors were challenged with tail vein injections of tumors to determine whether the presence of LN metastases facilitates distant metastasis. Through flow cytometry, single-cell RNA-seq, CRISPR/Cas9 knockout, adoptive transfer, model antigen, and cell depletion studies, we interrogate the cellular and molecular mechanisms mediating these processes.

Results We show that the presence of LN metastases promotes distant organ metastases, and this effect is eliminated in mice lacking adaptive immunity. Using flow cytometry and single-cell sequencing to perform comprehensive immune profiling, we identify multiple cellular mediators of tolerance. In particular, we find that LN metastases both resist NK cell cytotoxicity and induce regulatory T cells (Tregs). Depletion of NK cells *in vivo* enables non-metastatic tumors to disseminate to LNs, and ablation of Tregs using FoxP3-DTR mice eliminates the occurrence of lymphatic metastases. Adoptive transfer of Tregs from the LNs of mice bearing LN metastasis to naïve mice facilitates metastasis. We find that tumor specificity of the Tregs is necessary for the induction of systemic tolerance and the promotion of distant metastasis. Whole exome sequencing revealed that neither the metastatic proclivity nor immunosuppression evolve through the acquisition of driver mutations, loss of neoantigens, loss of MHC class I, or decreased melanoma antigen expression. Rather, by RNA-seq and ATAC-seq, we identify a conserved interferon signaling axis in LN metastases that is rendered stable through epigenetic reprogramming of chromatin accessibility resulting from chronic exposure to interferons. Using CRISPR/Cas9, we find that these pathways are required for LN metastasis, and validate their conserved significance in additional mouse models and humans with metastatic disease.

Conclusions Together, these findings demonstrate a critical role for LN metastasis in promoting tumor-specific immune tolerance. We previously found that immunotherapy efficacy is reliant upon immune activation within LNs, and we discuss strategies for overcoming tolerance to generate effective anti-tumor immunity.

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