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**PROFILING THE EFFECTS OF TARGETED TLR9 STIMULATION WITHIN SPONTANEOUSLY ARISING BREAST TUMORS**

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**Background** Tumor-localized delivery of immune-stimulants, such as Toll-like receptor (TLR) agonists, is a promising strategy to treat various cancer types, including immunologically “cold” tumors that lack T cell infiltration. Immunostimulants can elicit anti-tumor immunity by promoting immune activation in the tumor microenvironment (TME), thereby counteracting tumor immunosuppression and driving adaptive responses against tumor antigens available at the tumor site. However, the majority of preclinical studies investigating these therapies are performed in implanted murine tumor models, which do not fully recapitulate the TMEs of naturally-arising tumors. In this work, we performed comprehensive immune profiling studies to understand how tumor-localized TLR9 stimulation remodels the TME of spontaneously-arising tumors during an effective anti-tumor immune response.

**Methods** Serving as an immunologically “cold” spontaneous cancer model, we utilized female MMTV-PyMT transgenic mice, which spontaneously develop multiple breast tumors throughout their lives. To enable immunostimulant delivery to every tumor site, we employed a systemically-administered tumor-targeting immunostimulant (PIP-CpG), which is comprised of a tumor-targeting peptide (PIP) conjugated to an immune-stimulating TLR9 agonist (CpG).<sup>1</sup> This fully synthetic PIP-CpG conjugate is cross-reactive between mouse and human receptors and enables targeting to most types of solid tumors. To investigate how PIP-CpG therapy modulates the TME, we evaluated cytokine/chemokine profiles in the tumors via Luminescence analysis (48-plex) and performed spectral flow cytometry (24-color) to characterize various tumor-infiltrating immune populations and their activation status.

**Results** We demonstrate tumor-localized TLR9 stimulation via PIP-CpG therapy elicited a systemic anti-tumor immune response that effectively inhibited tumor growth and prolonged survival in an aggressive autochthonous breast cancer model. Immune profiling studies revealed that systemic PIP-CpG treatment dramatically amplified chemokine and cytokine production in the TME and promoted recruitment and expansion of many innate and adaptive immune cells. In addition to transforming the cellular landscape of the TME, PIP-CpG therapy also elicited diverse immune phenotypes that indicate changes in immune activation, cellular maturation, antigen presentation capacity, and inhibitory checkpoint expression. These immune-modulating effects ultimately enabled a T cell-mediated immune response against shared tumor antigens, which promoted regression of existing tumors and also delayed growth of independent newly-arising tumors.

**Conclusions** Intravenous delivery of a tumor-targeted TLR9 agonist transforms the immune microenvironment of spontaneously-arising malignancies to enable effective T cell-mediated immunity.

**REFERENCE**

1. Miller CL, Sagiv-Barfi I, Neuhöfer P, *et al.* Systemic delivery of a targeted synthetic immunostimulant transforms the immune landscape for effective tumor regression. *Cell Chem Biol* 2022;**29**:451–462.e8. doi:10.1016/j.chembiol.2021.10.012

**Ethics Approval** All mouse experiments were performed in accordance with protocols approved by the Stanford Administrative Panel on Laboratory Animal Care.

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