Werewolf THERAPEUTICS

Shifting the Balance In Cytokine Therapeutics

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Spatial Analysis of Tumor Infiltrating Lymphocyte Populations in Syngeneic Mouse Tumor Models After Treatment with IL-12 (mWTX-330) or IL-2 (WTX-124) INDUKINE™ Molecules

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BACKGROUND

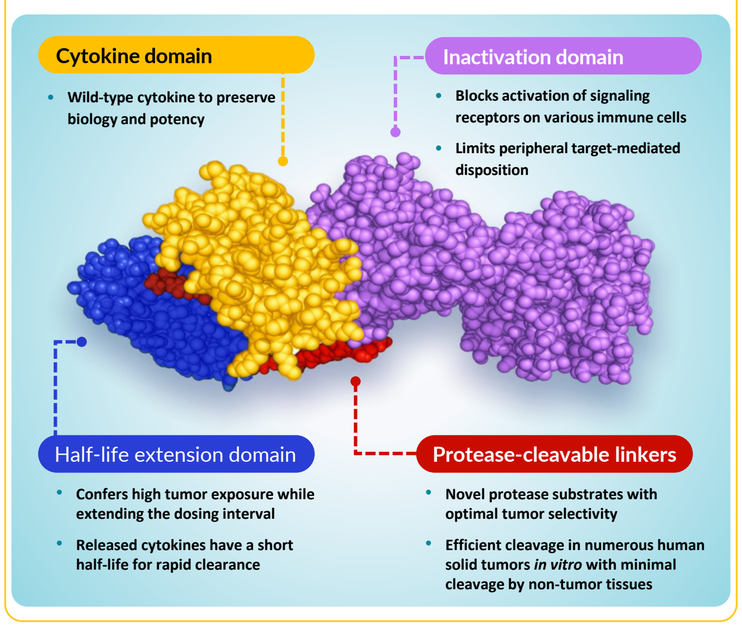
INDUKINE™ Molecules to Harness the Potential of Cytokines

Cytokines are powerful modulators of the immune system, making them a promising target for novel cancer immunotherapies. IL-12 is a pleotropic cytokine that acts on various immune cell populations, including professional APCs as well as adaptive and innate effector cells, making it an attractive molecule for cancer immunotherapy. Likewise, IL-2 is a potent activator of NK and T cell proliferation and effector function that has been approved for clinical use in melanoma and renal cell carcinoma. However, the wider use of cytokines in the clinic has been impeded by their poor pharmacokinetic properties and the serious adverse events associated with their systemic administration. To address these shortcomings, Werewolf Therapeutics has designed WTX-330[1] and WTX-124[2], selectively inducible cytokines (INDUKINE molecules) that contain wildtype cytokines, IL-12 and IL-2 respectively, linked to a half-life extension domain and an inactivation domain, by tumor selective protease cleavable linkers. Since human IL-12 is not active in murine models, a WTX-330 surrogate molecule containing chimeric IL-12, mWTX-330, was utilized for preclinical analysis.

Recent technical advances in multiplex immunofluorescence have highlighted the importance of spatial analysis in cancer immunotherapy. Uniquely, this approach allows for the detection and analysis of immune cell clustering and activation within the tumor microenvironment (TME), which a growing body of literature suggests is important for the development of an anti-tumor immune response. While traditional immunofluorescence techniques are often limited by a small number of markers or small regions of interest, the Lunaphore COMET™ system allows for spatial analysis of up to 40 markers simultaneously, without restricting the analysis to small regions of interest (ROIs). Using this instrument, we examined the effects of treatment of mWTX-330 on the TME in the EMT6 tumor model. Furthermore, we also assessed the effects of WTX-124, αPD-1, or the combination on the TME in the CT26 tumor model.

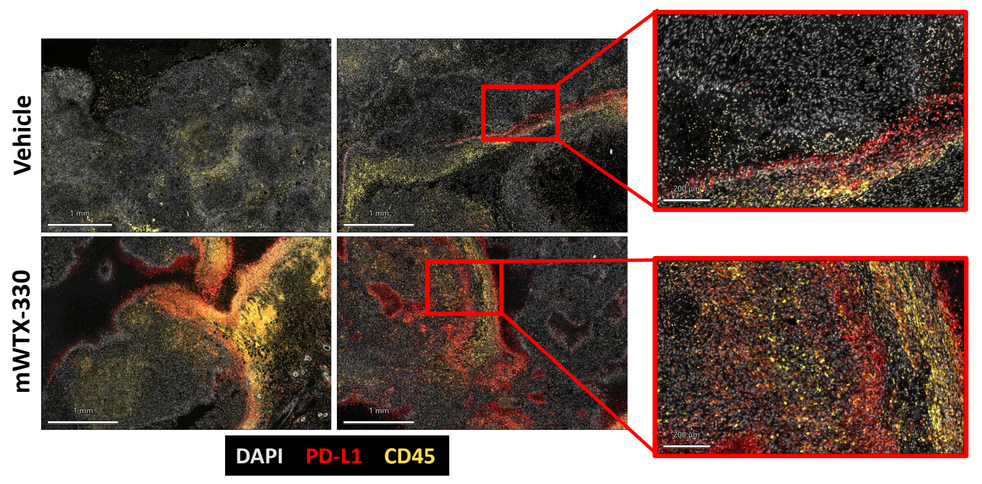
General Overview of INDUKINE Molecules

Addressing the Shortcomings of Cytokine Therapy



mWTX-330 Significantly Increases Tumor Infiltration by Immune Cells

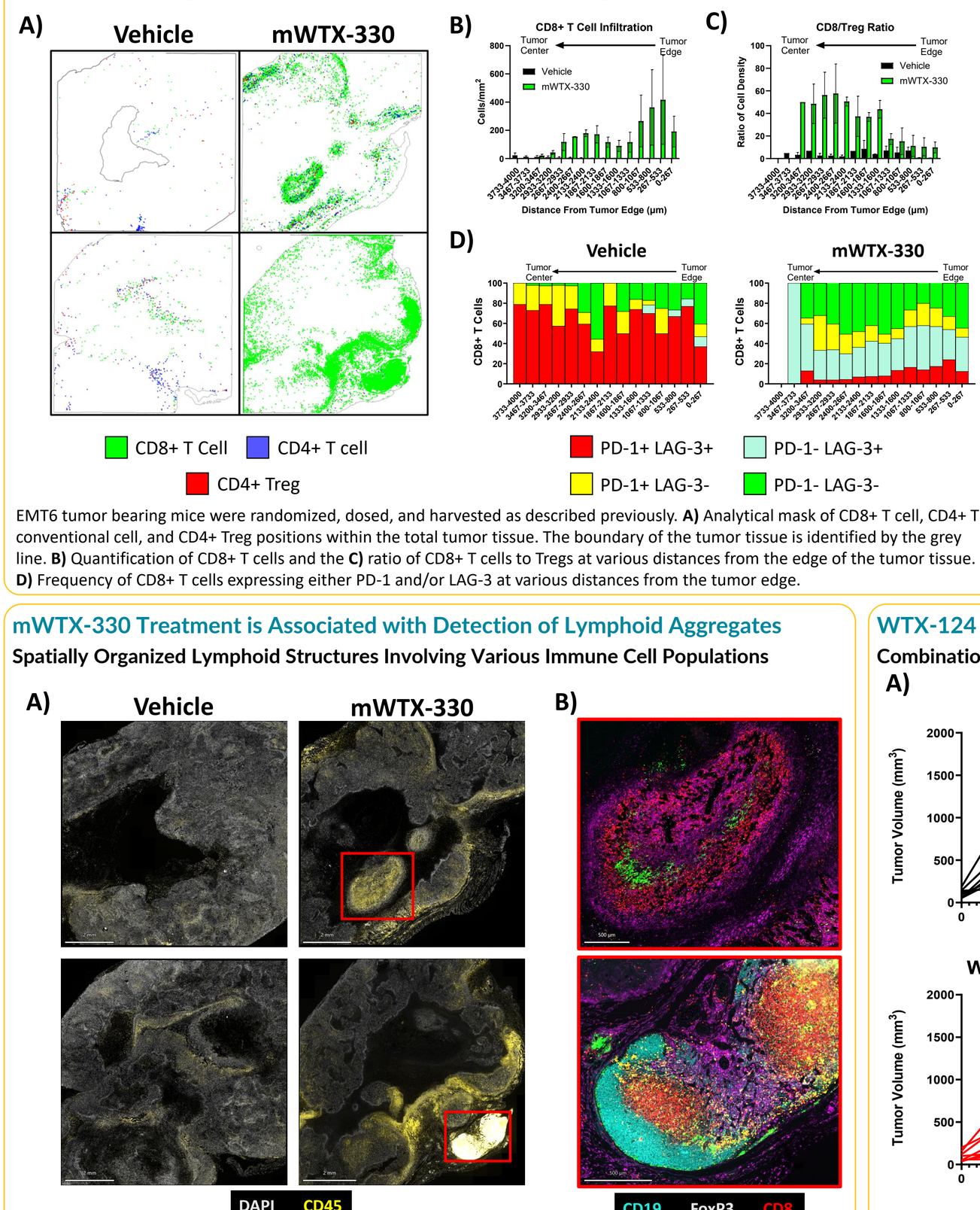
Chimeric IL-12 INDUKINE Molecule Drives Immune Cells Beyond PD-L1 Barrier



EMT6 tumor bearing mice were randomized into treatment groups and dosed twice a week for two weeks with mWTX-330, an INDUKINE molecule containing chimeric (human/mouse) IL-12 as a payload. On day 11, tumors were harvested, fixed in formalin, and FFPE blocks were generated for multiplex immunofluorescence staining. Examined markers are identified in the figure.

mWTX-330 Treatment Protects Tumor Infiltrating CD8+ T Cells from Exhaustion

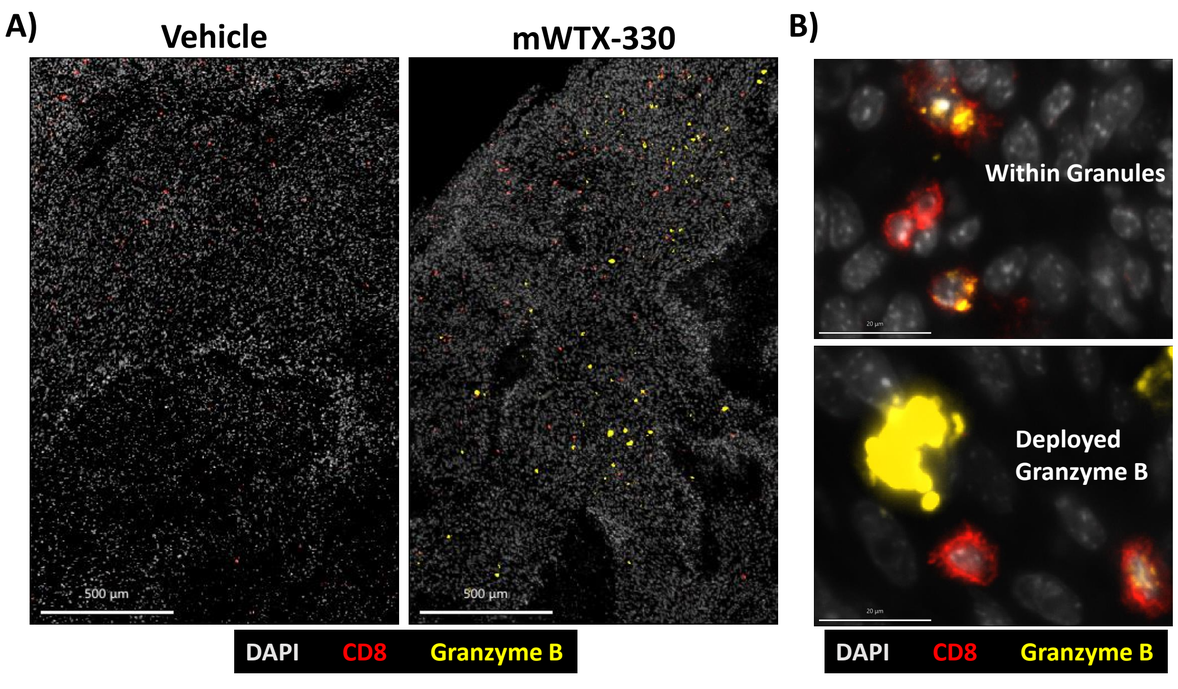
Increased CD8/Treg Ratio and Decreased PD-1 Expression Throughout the Tumor Tissue



EMT6 tumor bearing mice were randomized, dosed, and harvested as described previously. **A)** Analytical mask of CD8+ T cell, CD4+ T conventional cell, and CD4+ Treg positions within the total tumor tissue. The boundary of the tumor tissue is identified by the grey line. **B)** Quantification of CD8+ T cells and the **C)** ratio of CD8+ T cells to Tregs at various distances from the edge of the tumor tissue. **D)** Frequency of CD8+ T cells expressing either PD-1 and/or LAG-3 at various distances from the tumor edge.

mWTX-330 Activates Tumor Infiltrating Cytotoxic Effector Cells

Widespread Deployment of the Tumor Killing Effector Molecule Granzyme B Following Treatment



EMT6 tumor bearing mice were randomized, dosed, and harvested as described previously. **A)** Representative images demonstrating increased Granzyme B deployment throughout the tumor and the patterns of Granzyme B staining observed, including staining within individual granules as well as staining of released Granzyme B.

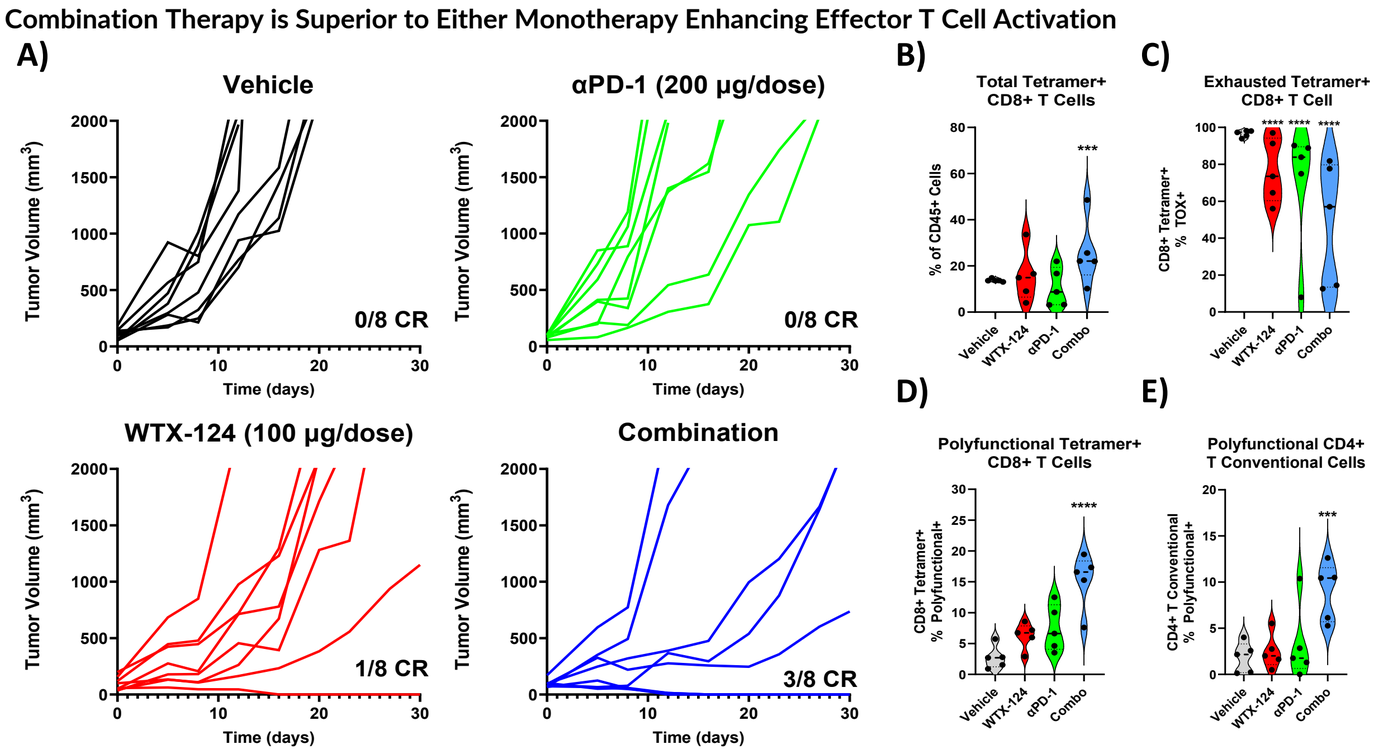
mWTX-330 Treatment is Associated with Detection of Lymphoid Aggregates

Spatially Organized Lymphoid Structures Involving Various Immune Cell Populations

EMT6 tumor bearing mice were randomized, dosed, and harvested as described previously. Examined markers are reported in the figure panels. **A)** Total tissue image identifying of zones with high density of CD45+ cells within the tumor. **B)** Magnified image of the high-density zones utilizing select markers to identify the various immune cell populations present and their locations in relation to each other.

WTX-124 Combined with αPD-1 Generates Robust Anti-Tumor Immunity in the CT26 Model

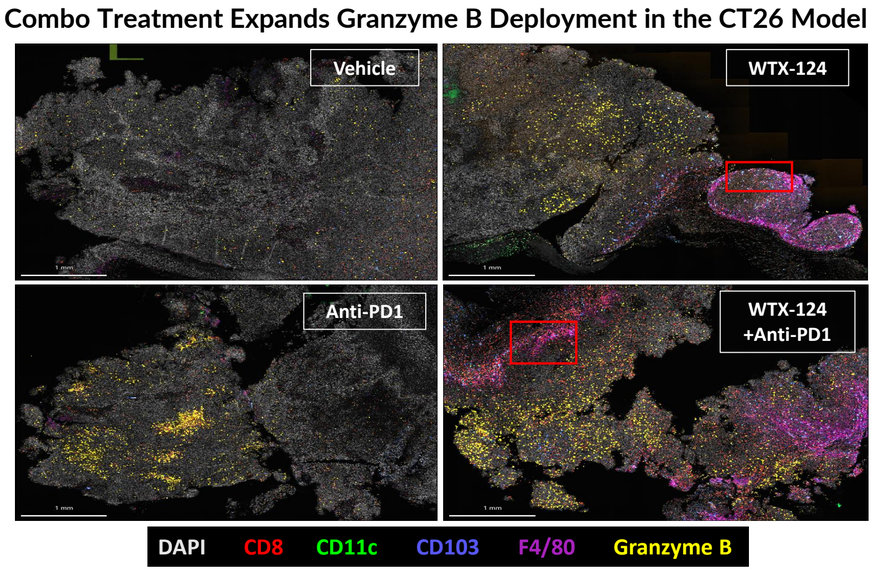
Combination Therapy is Superior to Either Monotherapy Enhancing Effector T Cell Activation



CT26 tumor bearing mice were randomized into treatment groups and dosed twice a week for one week with either WTX-124 (a wildtype IL-2 containing INDUKINE molecule), αPD-1, or the combination (doses noted in the figure). **A)** Tumor volume was monitored over time. Tumors were harvested on day 11 and TILs were analyzed by flow cytometry for **B)** the total frequency of tetramer+ CD8+ T cells, and **C)** the frequency of tetramer+ CD8+ T cells expressing the exhaustion marker TOX. TILs were also examined by intracellular cytokine staining for the frequency of **D)** tetramer+ CD8+ T cells or **E)** CD4+ T conventional cells producing two or more effector cytokines at once (polyfunctional). Statistics were generated using a two-way ANOVA, and significance is reported as follows: *** = p<0.001; **** = p<0.0001

Combination Treatment Drives Immune Activation in the TME

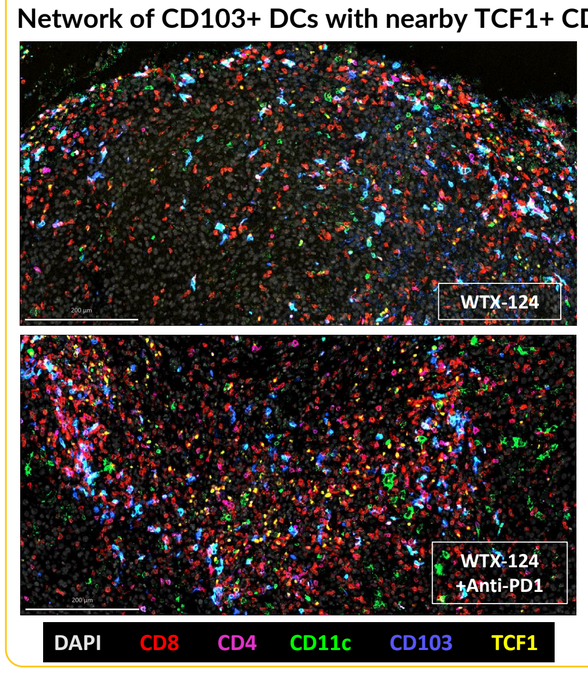
Combo Treatment Expands Granzyme B Deployment in the CT26 Model



CT26 tumor bearing mice were randomized, dosed, and harvested as described previously and examined by immunofluorescence. Examined markers are reported in the figure. Red squares indicate zones examined at higher magnification in the following figure.

Lymphoid Aggregates Correlate with WTX-124 Treatment

Network of CD103+ DCs with nearby TCF1+ CD8+ T Cells



CT26 tumor bearing mice were randomized, dosed, and harvested as described previously. Examined markers are reported in the figure. Red squares in the previous figure document the location of these lymphoid aggregates within the total tumor tissue. Figure documents areas where immune cells are congregating without Granzyme B deployment (as shown in the previous figure).

SUMMARY and REFERENCES

- Multiplex immunofluorescence is a powerful analysis technique that reveals additional spatial characteristics of immune activation following therapeutic intervention, which would be missed by low plex immunofluorescence, immunohistochemistry, and flow cytometry.
- mWTX-330 treatment results in deep infiltration of CD8+ T cells within the TME and widespread Granzyme B deployment while protecting those cells from exhaustion.
- Combination treatment with WTX-124 and αPD-1 also results in widespread tumor infiltration by CD8+ T cells and deployment of Granzyme B throughout the tissue.
- Treatment with INDUKINE molecules is associated with the detection of structured and unstructured lymphoid aggregates, which includes the clustering of various adaptive and innate immune cells within the TME. These zones are typically devoid of effector cytokine deployment, suggestive of a zone of cytotoxic cell education within the TME.

1. Nirschl, C. J. et al. mWTX-330, an IL-12 INDUKINE Molecule, Activates and Reshapes Tumor-Infiltrating CD8 T and NK Cells to Generate Antitumor Immunity. Cancer Immunol. Res. 11, 962–977 (2023).
2. Nirschl, C. J. et al. Discovery of a Conditionally Activated IL-2 that Promotes Antitumor Immunity and Induces Tumor Regression. Cancer Immunol. Res. 10, 581–596 (2022).

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