Werewolf THERAPEUTICS

Shifting the Balance In Cytokine Therapeutics

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Abstract # 4078

WTX-712, a Conditionally Active IL-21 INDUKINE™ Molecule, Induces a Strong Anti-tumor Phenotype Through a Differentiated Mechanism

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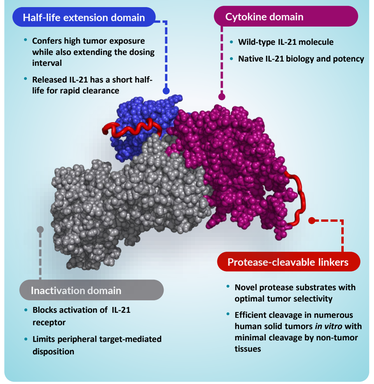
BACKGROUND

Development of WTX-712, an IL-21 Prodrug for Cancer Immunotherapy

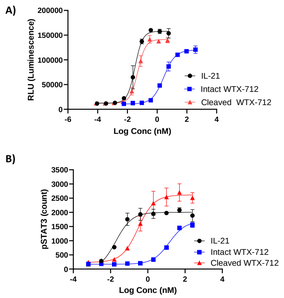
Despite great strides seen recently in the development of immunotherapies such as immune checkpoint inhibitors (ICI), many patients do not respond or acquire resistance, highlighting a need for alternative immunotherapies. As potent immunomodulators, cytokines have been explored as treatments for cancer, but their use has been limited due to toxicity and poor pharmacokinetics (PK). One of these key cytokines, interleukin 21 (IL-21), is a pluripotent cytokine that activates anti-tumor T cell responses, induces B cell activation, and promotes generation and maintenance of germinal centers and tertiary lymphoid structures. IL-21 acts on a broader range of cells and does not induce vascular leak syndrome compared to IL-2, another common-gamma chain family member. Half-life extended IL-21 (IL-21-HLE) drives robust anti-tumor activity in several murine syngeneic tumor models. Of particular interest, IL-21-HLE showed superior anti-tumor activity compared to IL-2-HLE in the EMT-6 and Renca models, both of which are highly resistant to anti-PD-1/PD-L1 treatment. Although IL-21-HLE and IL-2-HLE induced a similar frequency of tumor infiltrating CD8+ T cells, IL-21-HLE treatment led to higher and longer lasting polyfunctionality (co-expression of effector molecules) in CD8+ T cells. Of note, IL-21-HLE induced greater amounts of effector cytokines, Granzyme B and Perforin, than IL-2-HLE. Anti-tumor efficacy was linked to expansion and activation of tumor infiltrating CD8+ T cells with increased polyfunctionality. Transcriptomic analysis revealed that IL-21 drives upregulation of a type I IFN signature in the tumor, promoting an anti-tumorigenic microenvironment. Clinical activity of IL-21 has been hampered by poor PK and adverse events at dose levels associated with signs of efficacy. To overcome these limitations, Werewolf Therapeutics has developed WTX-712, an IL-21 INDUKINE™ molecule, which contains wild-type human IL-21, an inactivation domain, and a half-life extension domain tethered together by protease sensitive linkers. In preclinical studies with mouse syngeneic tumor models, WTX-712 is inactive in the periphery, and fully active IL-21 is selectively released within the tumor, resulting in anti-tumor efficacy with an expanded therapeutic window compared to IL-21-HLE.

Key Features of INDUKINE Molecules



WTX-712 is an Inducible IL-21 Pro-Drug

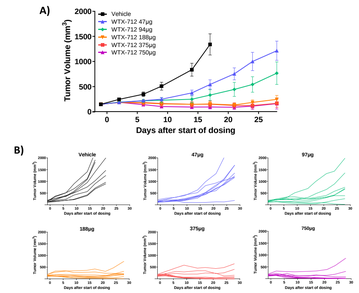
In Vitro Cellular Data



***(A)** In vitro activity of WTX-712 in the IL-21 Promega reporter assay or **(B)** measurement of pSTAT3 in human PBMCs after incubation comparing intact WTX-712 (blue squares) and in vitro cleaved/activated WTX-712 (red triangles) to recombinant IL-21 (black circles).*

Treatment with WTX-712 Results in Robust Anti-Tumor Activity

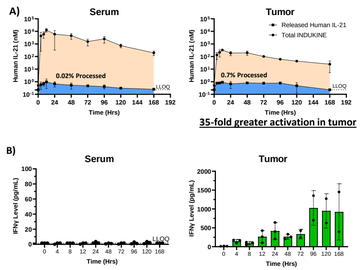
Dose Dependent Efficacy of WTX-712



*hIL-21RKI mice bearing MC38 tumors were randomized into treatment groups, dosed twice a week with WTX-712 for two weeks, and tumors were measured twice weekly. **(A)** Average tumor volume for n=8 animals per group and **(B)** individual spider plots of tumor burden.*

Favorable WTX-712 Exposure and Tumor Activation

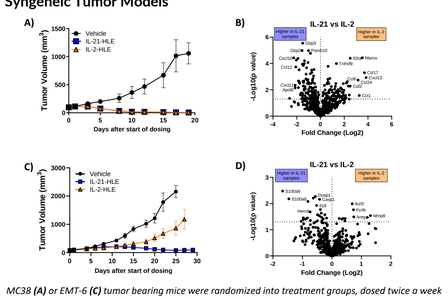
WTX-712 is Preferentially Processed in the Tumor Microenvironment



*hIL-21RKI MC38 tumor bearing mice were randomized into treatment groups and dosed once with WTX-712. Mice were sacrificed at indicated timepoints, and serum and tumors were collected. **(A)** Total WTX-712 and free IL-21 exposure. **(B)** IFNγ generation in serum and tumor after single dose of WTX-712.*

IL-2 and IL-21 Cytokines Trigger Different Responses in Tumor Models

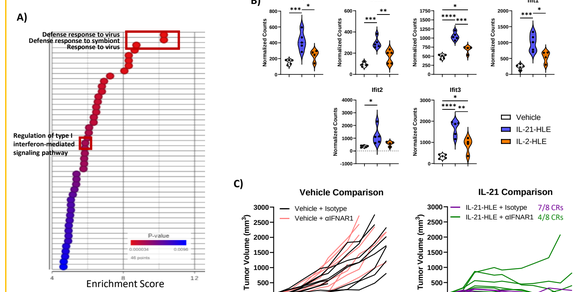
Differential Efficacy of IL-2 and IL-21 in Anti-PD-1 Resistant Syngeneic Tumor Models



*MC38 **(A)** or EMT-6 **(C)** tumor bearing mice were randomized into treatment groups, dosed twice a week for two weeks with vehicle (black), IL-2-HLE (red) or IL-21-HLE (blue). Tumor volumes were measured twice a week. Nanostring analysis of RNA from MC38 **(B)** or EMT-6 **(D)** tumors after dosing with IL-2-HLE or IL-21-HLE. Differentially expressed genes between IL-2-HLE and IL-21-HLE samples graphed as volcano plot comparing fold change and p value.*

IL-21 Activates Type I IFN Signaling in the TME

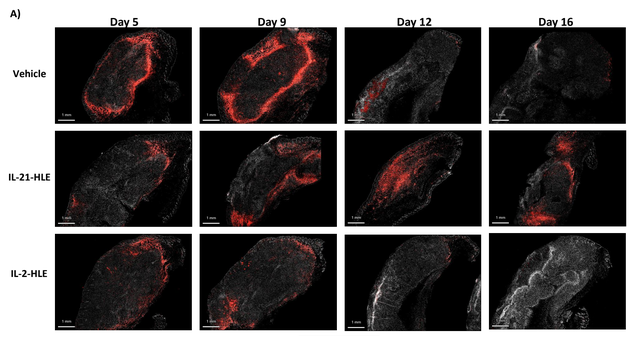
Type I IFN Pathway Upregulated after IL-21 Treatment, and IFNAR1 Blockade Reduces IL-21 Efficacy



***(A)** EMT-6 tumor bearing mice were randomized into treatment groups (Day 0), dosed twice a week with IL-21-HLE or IL-2-HLE for two weeks, and tumors were harvested for Nanostring analysis of bulk RNA. Pathway analysis preformed on the differentially expressed genes and graph shows pathways identified which were significantly enriched with a p value <0.05 and enrichment greater than 3-fold. **(B)** Plots of genes identified in IFN related pathways. p values represent the results of a one-way ANOVA: * = p<0.05, ** = p<0.005 ***= p<0.001, ****= p<0.0001 **(C)** EMT-6 tumor bearing mice were randomized into treatment groups (Day 0), mice were dosed every third day with anti-IFNAR1 or isotype control for a total of 8 doses. Mice were dosed twice a week with IL-21-HLE or vehicle for two weeks. Tumors and body weight were measured twice weekly.*

Spatial Profiling Reveals Differential Intratumoral Immune Infiltration after IL-21-HLE Treatment Compared to IL-2 HLE

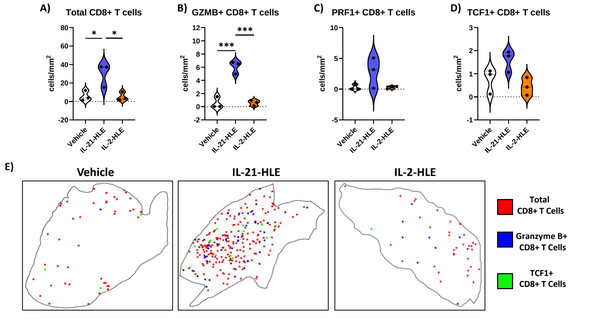
IL-21-HLE Treatment Results in Enhanced and Sustained Immune Infiltration Over Time



*EMT-6 tumor bearing mice were randomized into treatment groups and dosed twice weekly for two weeks with either IL-21-HLE or IL-2-HLE. Tumors were collected indicated timepoint and fixed in formalin. Tumors were embedded in paraffin, sectioned, dewaxed and antigen retrieved. Tumors were stained and imaged using a Lunaphore COMET multiplex immunofluorescence platform. Representative images showing staining for **(A)** DAPI and CD45 at various timepoints. **(B)** DAPI, CD8, CD4, F4/80, CD11b and Granzyme B at day 12 **(C)** DAPI, CD8, CD4, TCF1, CD11c, CD103 and NCR1 at day 12*

IL-21 Promotes Increased Polyfunctional CD8+ T cells in the Tumor

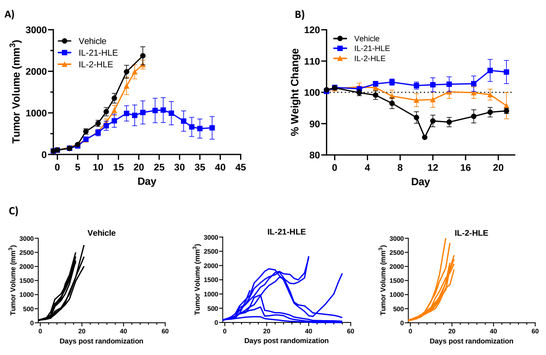
Quantification of Spatial Profiling Shows Increased Activated CD8+ T cells with IL-21 Treatment



*EMT-6 tumor bearing mice were randomized into treatment groups and dosed twice weekly for two weeks with either IL-21-HLE or IL-2-HLE. Tumors were collected at day 12 and fixed in formalin. Tumors were embedded in paraffin, sectioned, dewaxed and antigen retrieved. Tumors were stained and imaged using a Lunaphore COMET multiplex immunofluorescence platform. Three individual tumors were imaged and analyzed per group. Images were analyzed using HALO Software from Indica Labs, and indicated cell populations quantified and normalized to tissue area. Quantification of **(A)** Total CD8+ T cells, **(B)** Granzyme B+ CD8+ T cells, **(C)** Perforin+ CD8+ T cells and **(D)** TCF1+ CD8+ T cells. p values represent the results of a one-way ANOVA: * = p<0.05, ***= p<0.001. **(E)** Spatial dot plot of CD8+ T cell infiltration of tumor tissue.*

Treatment with IL-21 Results in Robust Anti-Tumor Efficacy in CPI Resistant Tumor Model

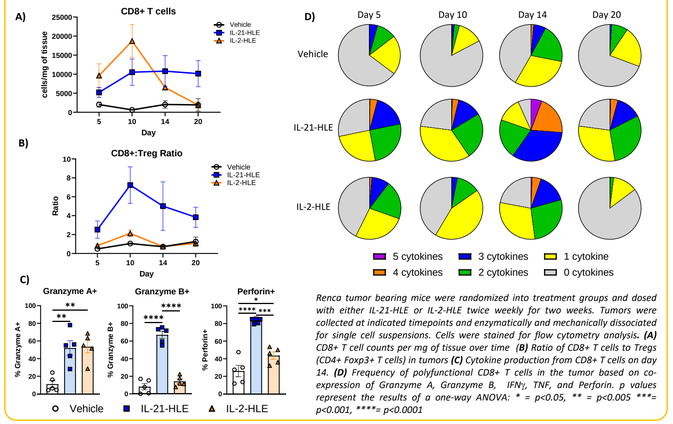
Differential Activity of IL-21 and IL-2 in Renca Tumor Model



*Renca tumor bearing mice were randomized into treatment groups and dosed with either IL-21-HLE or IL-2-HLE twice weekly for two weeks. **(A)** Tumors and **(B)** body weight were measured twice weekly. **(C)** Individual spider plots of tumor burden with extended monitoring for IL-21-HLE treated mice.*

IL-21 Treatment Leads to Sustained Polyfunctional CD8+ T cell Population

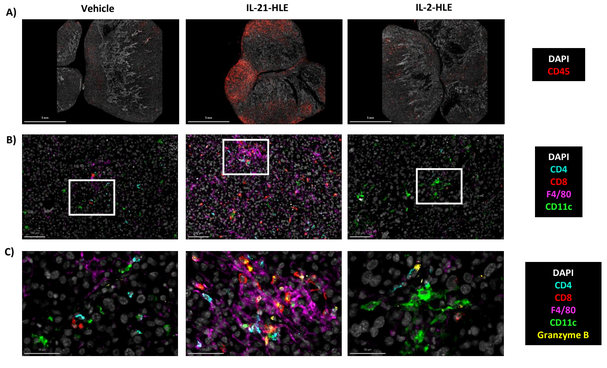
IL-21 Promotes Cytotoxic CD8+ T cells Expressing High Levels of Granzymes and Perforin



*Renca tumor bearing mice were randomized into treatment groups and dosed with either IL-21-HLE or IL-2-HLE twice weekly for two weeks. Tumors were collected at indicated timepoints and enzymatically and mechanically dissociated for single cell suspensions. Cells were stained for flow cytometry analysis. **(A)** CD8+ T cell counts per mg of tissue over time **(B)** Ratio of CD8+ T cells to Tregs (CD4+ Foxp3+ T cells) in tumors **(C)** Cytokine production from CD8+ T cells on day 14. **(D)** Frequency of polyfunctional CD8+ T cells in the tumor based on co-expression of Granzyme A, Granzyme B, IFNγ, TNF, and Perforin. p values represent the results of a one-way ANOVA: * = p<0.05, ** = p<0.005 ***= p<0.001, ****= p<0.0001*

Spatial Profiling Shows Robust Infiltration of Immune Cells after IL-21 Treatment

IL-21 Treatment Leads to Increased CD8+ T cells and Immune Hubs in TME



*Renca tumor bearing mice were randomized into treatment groups and dosed with either IL-21-HLE or IL-2-HLE twice weekly for two weeks. Tumors were collected at day 14 after start of dosing and fixed in formalin. Tumors were embedded in paraffin, sectioned, dewaxed and antigen retrieved. Tumors were stained and imaged using a Lunaphore COMET multiplex immunofluorescence platform. **(A)** DAPI and CD45 **(B)** DAPI, CD4, CD8, F4/80, CD11c, **(C)** Closer magnification of the white boxed area in corresponding images in (B), DAPI, CD4, CD8, F4/80, CD11c, and Granzyme B.*

SUMMARY and CONCLUSIONS

- WTX-712 is a novel INDUKINE molecule engineered to enhance the therapeutic window of IL-21
- WTX-712 demonstrates *in vitro* inducibility and activity
- WTX-712 is inactive in the periphery and shows selective release of free IL-21 in the TME linked to IFNγ production
- Anti-tumor efficacy driven by IL-21 differs from that of other potent pro-inflammatory cytokines such as IL-2
- IL-21 shows superior anti-tumor activity compared to IL-2 in CPI resistant EMT-6 and Renca syngeneic tumor models
- IL-21 promotes a type I IFN signature in the TME, and blockade of IFNAR1 signaling reduces the efficacy of IL-21 anti-tumor activity
- IL-21 treatment drives a sustained cytotoxic CD8+ T cell polyfunctional population with increased expression of Granzymes and Perforin
- Together, these data support continued exploration of WTX-712, an IL-21 INDUKINE molecule, as a therapy for cancer

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