INDUKINE™ Molecules Delivering Various Cytokines Utilize Unique Mechanisms of Action to Drive Anti-Tumor Efficacy in a Murine Syngeneic Tumor Model

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Abstract # 955

Werewolf THERAPEUTICS

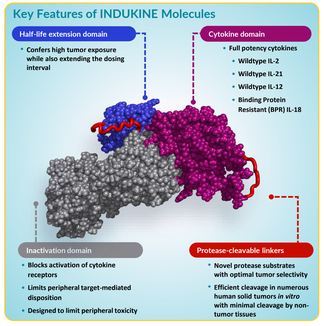
Shifting the Balance In Cytokine Therapeutics

BACKGROUND

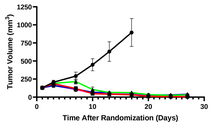
Cytokine Therapy For Immunotherapy

Cytokines play a dominant role in determining the potency and outcome of an immune response, making them an attractive target for novel cancer immunotherapies. However, recombinant cytokine therapies have broadly failed to live up to their potential, largely due to their poor PK properties and toxicity when administered systemically. To address these shortcomings, we have developed half-life extended, selectively inducible cytokine molecules named INDUKINE™ molecules, which have been engineered with a variety of cytokines to be preferentially activated in the tumor microenvironment (TME). IL-18 is an IL-1 superfamily cytokine that, in combination with other cytokines, induces IFNγ production by a variety of immune cell populations. Likewise, IL-12 is a pleotropic cytokine that acts not only on CD8+ T cells, but also activates natural killer cells and antigen presenting dendritic cells. Lastly, IL-21 and IL-2 are both cytokines of the common gamma chain family that can also generate antitumor immunity in murine syngeneic models, with potent activity on effector cell populations. While these cytokines can generate robust antitumor immunity as monotherapies, a direct head-to-head comparison within a single tumor model could elucidate key differences in their individual mechanisms of action. In this study, we utilized the MC38 murine syngeneic tumor model to explore the differential mechanisms of action utilized by these various cytokines.

Key Features of INDUKINE Molecules



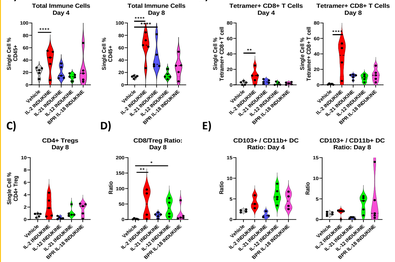
Tumor Rejection is Induced by all INDUKINE Molecules as Monotherapy



C57Bl/6 mice bearing MC38 tumors were randomized into treatment groups, dosed twice a week with a curative dose of INDUKINE molecules for two weeks, and tumors were measured twice weekly. Average tumor volume for n=8 animals per group. In some figures, tumors were harvested at indicated timepoints for flow cytometry, NanoString analysis, multiplex immunofluorescence, or multiomics analysis

INDUKINE Molecules Shape TME Infiltration

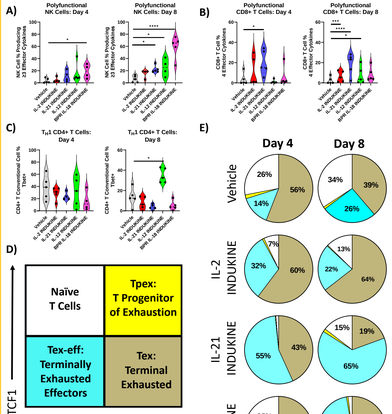
IL-2 Drives Significant Tetramer+ CD8+ T Cell Expansion



*C57Bl/6 mice bearing MC38 tumors were treated as previously described, and tumors were harvested for flow cytometry A) Total immune cell, B) tetramer+ CD8+ T cell, or C) CD4+ Treg frequency among total single cells. D-E) Ratio of D) tetramer+ CD8 T cells to Tregs or E) CD103+ dendritic cells to CD11b+ dendritic cells at the indicated time points. Statistics represent a two-way ANOVA with multiple comparisons, where: * = p<0.05, ** = p<0.01, **** = p<0.0001.*

INDUKINE Molecules Activate Tumor Infiltrating Immune Cells

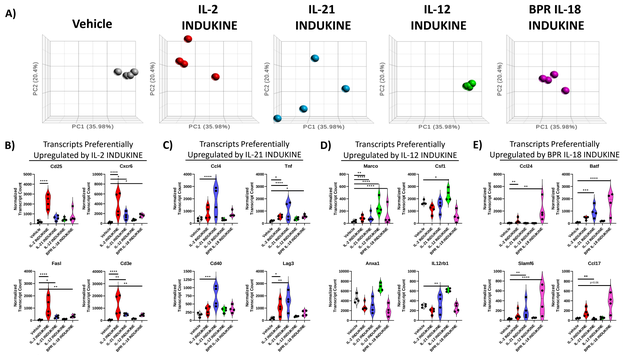
BPR IL-18 Drives a Tpex Phenotype in the CD8+ T Cells



*C57Bl/6 mice bearing MC38 tumors were treated as previously described, and tumors were harvested for flow cytometry. The frequency of polyfunctional (producing multiple effector cytokines out of IFNγ, TNF, Granzyme B, or Perforin) **A)** NK cells or **B)** CD8+ T cells, or **C)** Tbet+ CD4+ T conventional cells (T_H1). **D)** The molecular definitions of Naïve (TCF1+TOX-), Tpex (TCF+ TOX+), Tex-eff (TCF1-TOX-), and Tex (TCF1-TOX+), and **E)** the average frequency of those populations within the CD8+ T cell population. Statistics represent a two-way ANOVA with multiple comparisons, where: * = p<0.05, *** = p<0.001, **** = p<0.0001.*

INDUKINE Molecules Drive Unique Transcriptional Signatures

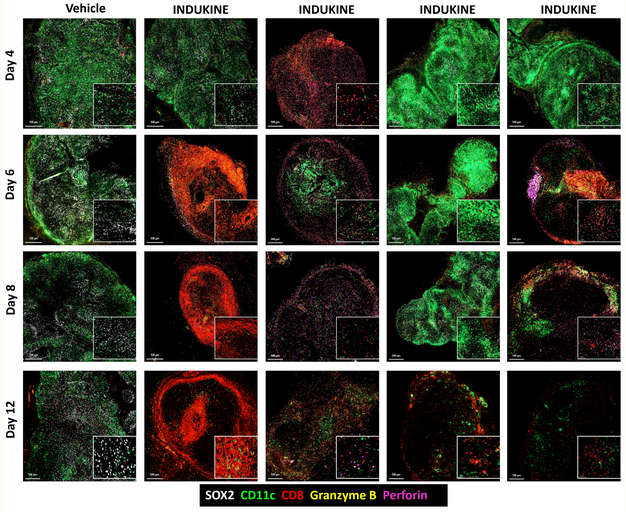
PCA Analysis Reveals Distinct Transcriptional Programming by Individual Cytokines



*C57Bl/6 mice bearing MC38 tumors were treated as previously described, and tumors were harvested on Day 8. Bulk RNA was extracted for NanoString analysis. A) PCA analysis of samples grouped by treatment. Representative transcripts that were preferentially upregulated by B) IL-2, C) IL-21, D) IL-12, or E) BPR IL-18 INDUKINE treatment. Statistics were generated using Partek DESeq2 analysis, where: * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.*

INDUKINE Molecules Activate Multiple, Unique Effector Mechanisms

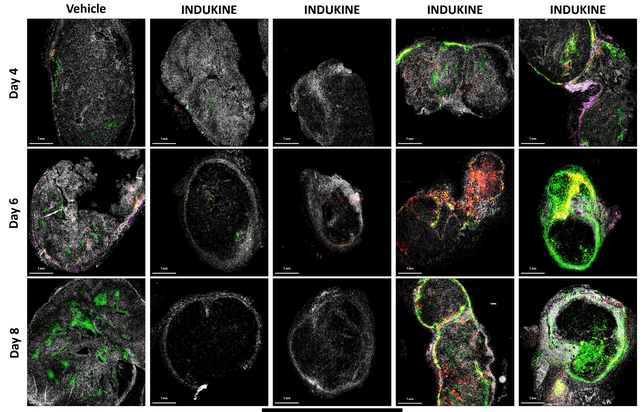
IL-2 and IL-21 Primarily Target CD8+ T Cells, While IL-12 and BPR IL-18 Also Recruit Dendritic Cells



C57Bl/6 mice bearing MC38 tumors were treated as previously described. Tumors were harvested for multiplex immunofluorescence at the indicated timepoints and fixed in formalin before being embedded in paraffin, sectioned, and dewaxed and antigen retrieved. Tumors were stained and imaged using a Lunaphore COMET multiplex immunofluorescence platform. Representative images showing staining for SOX2, CD11c, CD8, Granzyme B, and Perforin at the indicated timepoints.

IL-12 and IL-18 INDUKINE Molecules Drive Robust Induction of PD-L1 and iNOS

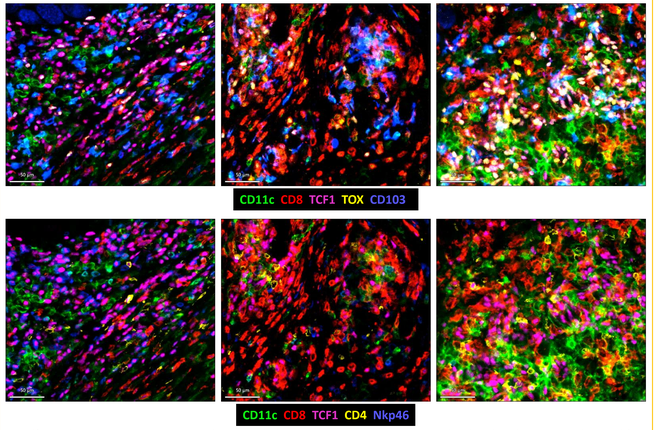
Unique Induction of Dense Zones of Cells Expressing TCF1 with BPR IL-18 INDUKINE Treatment



C57Bl/6 mice bearing MC38 tumors were treated as previously described. Likewise, tumors were harvested at the indicated timepoints and prepared for multiplex immunofluorescence as previously described. Tumors were stained and imaged using a Lunaphore COMET multiplex immunofluorescence platform. Representative images showing staining for DAPI, iNOS, PD-L1, and TCF1 at the indicated timepoints.

BPR IL-18 INDUKINE Drives the Accumulation of CD8+ Tpex Cells in Immune Hubs

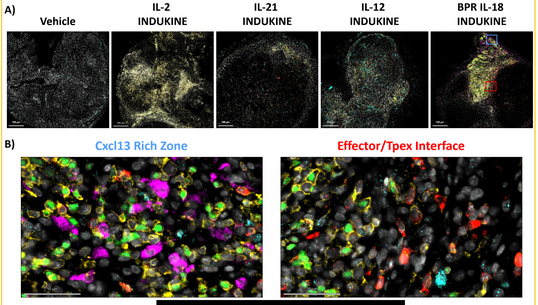
Early Generation and Maintenance of Immune Hubs with Dense TCF1+ CD8+ T Cell Neighborhoods



*C57Bl/6 mice bearing MC38 tumors were treated as previously described. Likewise, tumors were harvested at the indicated timepoints and prepared for multiplex immunofluorescence as previously described. Tumors were stained and imaged using a Lunaphore COMET multiplex immunofluorescence platform. Representative images showing staining for either **A)** CD11c, CD8, TCF1, TOX, and CD103 or **B)** CD11c, CD8, TCF1, CD4, and Nkp46 at the indicated timepoints.*

Multiomics Analysis Reveals Cxcl13 Induction by BPR IL-18 INDUKINE

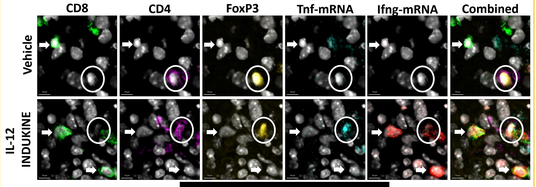
Cxcl13 Expression Found on Non-Immune Cells In the Immune Hubs



*C57Bl/6 mice bearing MC38 tumors were treated with the BPR IL-18 INDUKINE, and tumors were harvested on Day 6 post initiation of treatment and prepared for multiplex immunofluorescence as previously described. These data were collected using an un-released version of the COMET multiomics platform and software and may not represent the final system available upon official release. Representative images showing protein staining for CD45 and TCF1, and RNA-Scope staining for the transcripts associated with murine IFNγ, TNFα, and CXCL13 at either **A)** low magnification or **B)** high magnification.*

IL-12 INDUKINE Treatment Induces Fragility Phenotype in CD4+ Tregs

Induction of Ifng and Tnf Transcripts in FOXP3+ T Cells



C57Bl/6 mice bearing MC38 tumors were treated and harvested as previously described on Day 6. Data were collected using an un-released version of the COMET multiomics platform as stated in the previous figure. Representative images showing protein staining for CD8, CD4, FoxP3, and RNA-Scope staining for the transcripts associated with murine IFNγ and TNFα. Images reveal that IL-12 INDUKINE treatment results in FoxP3+ Tregs transcribing the mRNA associated with effector cytokine production as we previously reported[1] using flow analysis. White arrows (CD8+ T cells) and white circles (CD4+ Tregs) highlighted in the images.

CONCLUSIONS

- All the tested INDUKINE molecules resulted in complete tumor rejections in the murine syngeneic tumor model MC38.
- Treatment with IL-2 INDUKINE resulted in substantial CD8+ T cells expansion in response to treatment, while treatment with IL-12 INDUKINE resulted in an increase in the CD103+/CD11b+ dendritic cell ratio.
- Treatment with IL-21 INDUKINE resulted in the greatest frequency of polyfunctional CD8+ T cells, while BPR IL-18 INDUKINE treatment resulted in the greatest frequency of polyfunctional NK cells and the induction of Tpex cells within the TME.
- IL-12 and BPR IL-18 INDUKINE treatment resulted in PD-L1 and iNOS upregulation
- BPR IL-18 INDUKINE treatment resulted in the establishment of immune hubs, including CD4+ T Cells, CD8+ Tpex T cells, dendritic cells, and NK cells
  - Immune hub formation correlated with the induction of Cxcl13 among non-immune cells within the immune hub
- IL-12 INDUKINE treatment induces a fragility phenotype in tumor infiltrating Tregs, driving production of the RNA transcripts for Ifng and Tnf
- Each cytokine utilizes specific, unique mechanisms of action to generate anti-tumor immunity, all of which may prove important in the clinic to generate robust anti-tumor responses in patients

[1]Nirschl, C. J., Brodkin, H. R., Domonkos, C., Dwyer, C. J., Hicklin, D. J., Ismail, N., Seidel-Dugan, C., Steiner, P., Steuert, Z., Sullivan, J. M., Winston, W. M., & Salmeron, A. (2023). mWTX-330, an IL-12 INDUKINE Molecule, Activates and Reshapes Tumor-Infiltrating CD8b T and NK Cells to Generate Antitumor Immunity. *Cancer Immunology Research, 11*(7), 962–977.

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