A half-life extended, tumor selective IL-12 activated tumor infiltrating immune cells and demonstrated anti-tumor activity in the MC38 syngeneic mouse model

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Introduction

Interleukin-12 (IL-12) is a proinflammatory cytokine that bridges innate and adaptive immunity via induction of T helper 1 differentiation and promoting cytolytic activity of natural killer, as well as T cells. IL-12 has demonstrated potent anti-tumor activity in syngeneic mouse models and promising anti-tumor activity in humans. However, clinical development of IL-12 has been limited by severe systemic toxicities. To overcome toxicity and improve the therapeutic index of IL-12 in a clinical setting, XTX301 was engineered as a potent, half-life extended and masked IL-12. The masking domain of XTX301 is designed to pharmacologically inactivate IL-12 in non-tumor tissue, while enabling generation of an active IL-12 moiety upon cleavage by proteases that are enriched in the tumor microenvironment.

Design and predicted activity of XTX301, a tumor selective IL-12

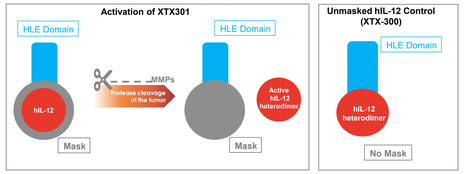


Figure 1: The masking domain of XTX301 is designed to pharmacologically inactivate IL-12 in non-tumor tissue and render an active IL-12 moiety upon cleavage by proteases that are enriched in the tumor microenvironment. XTX300 was designed to serve as an unmasked control molecule. Human IL-12 does not cross react with mouse IL-12 receptors; hence a murine surrogate (mXTX301) and unmasked control (mXTX302) were created to evaluate anti-tumor activity *in vivo*. HLE=Half-life extension domain MMP=matrix metalloproteinase

Masking of XTX301 IL-12 activity and reactivation by MMPs *in vitro*

XTX301 is pharmacologically active upon proteolytic cleavage in cell-based assays

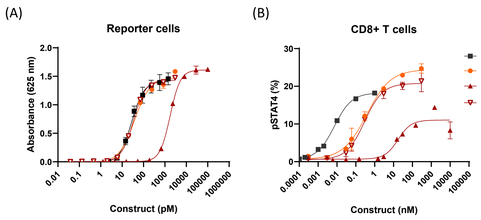


Figure 2: (A) IL-12 activity was measured with an IL-12 reporter gene cell line, which is engineered to express reporter upon IL-12-mediated STAT4 activation. Cells incubated with XTX301 demonstrated reduced reporter activity compared to cells treated with rhIL-12 or XTX300. Upon MMP activation, XTX301 demonstrated full potency. (B) Primary human peripheral blood mononuclear cells (PBMCs) were preactivated and then incubated with varying doses of rhIL-12 and test articles for 24 hours and evaluated for STAT4 phosphorylation by flow cytometry. XTX301 treatment resulted in attenuated STAT4 phosphorylation compared to rhIL-12 and XTX300. Upon MMP activation, XTX301 treatment resulted in a similar STAT4 phosphorylation as unmasked XTX300.

Anti-tumor activity of mXTX301 *in vivo*

mXTX301 demonstrated anti-tumor activity in MC38 syngeneic tumor model without body weight loss

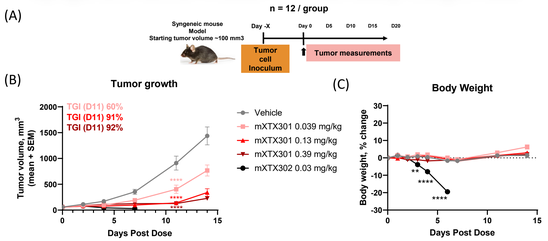


Figure 3: Evaluation of anti-tumor activity of mXTX301 in MC38 syngeneic tumor model.
(A) Animals were implanted subcutaneously with tumor cells and received a single intravenous injection of mXTX302 at a dose 0.03 mg/kg or mXTX301 at 0.039, 0.13, and 0.39 mg/kg. Tumor and body weight measurements were taken two/three times a week.
(B) mXTX301 demonstrated dose-dependent anti-tumor activity in the MC38 syngeneic tumor model with 2/12 and 1/12 tumor free mice in the groups treated with mXTX301 at 0.13 and 0.39 mg/kg, respectively. Tumor growth data are presented as mean for tumor volume ± SEM. Two-way ANOVA followed by Bonferroni post-hoc test (****p < 0.0001).
(C) mXTX302 was not tolerated and resulted in 20% body weight loss by Day 6. 75% of animals were euthanized by Day 11. Compared to vehicle control, mXTX301 was well tolerated in MC38 model with no significant body weight loss. Body weight % change mean ± SEM was shown. Two-way ANOVA followed by Bonferroni post-hoc test (**p < 0.005; ****p < 0.0001).

mXTX301 demonstrated effective peripheral masking in MC38 mouse model

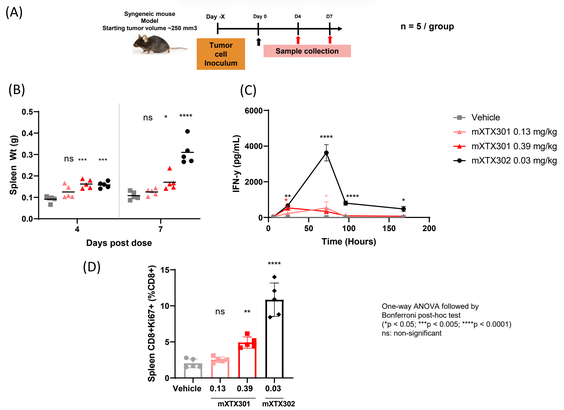


Figure 4: Evaluation of peripheral activity of mXTX301 in MC38 syngeneic tumor model. (A) Schematic of the pharmacodynamic study. (B) mXTX301 at 0.39 mg/kg resulted in moderate splenomegaly on Day 4 and Day 7. Unmasked 0.03 mg/kg mXTX302 treatment resulted in significant splenomegaly on Day 7. (C) mXTX301 at doses 0.13, and 0.39 mg/kg resulted in an increase of plasma IFNγ with a peak at 72 and 24 hours, respectively. Compared to masked mXTX301, mXTX302 resulted in 7-10-times higher IFNγ response at 72 hours. The level of cytokine remained high at 96 and 168 hrs. (D) Compared with control, mXTX301 at 0.39 mg/kg resulted in an increase in the percentage of proliferating CD8+Ki67+ T cells in spleen. mXTX302 increased the number of proliferating CD8+ cells 5.3-fold compared to the vehicle group.

Mechanism of action of mXTX301 *in vivo*

mXTX301 treatment resulted in increased presence of CD8 T cells in the tumor microenvironment (TME)

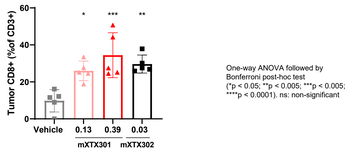
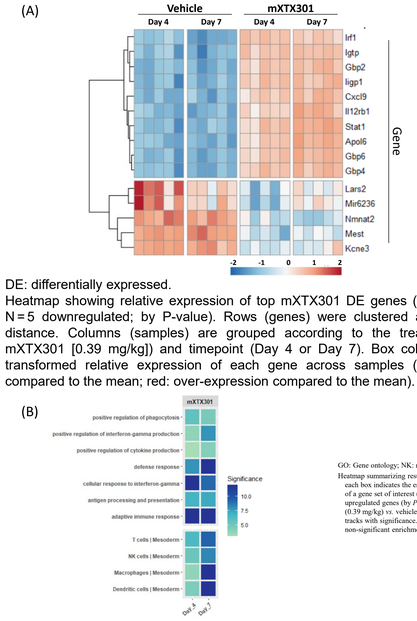


Figure 5: Effect of mXTX301 on MC38 tumor microenvironment. Mice dosed with mXTX301 at 0.13 and 0.39 mg/kg and with mXTX302 at 0.03 mg/kg induced infiltration of CD8+ T cells into tumor.

mXTX301 induced pro-inflammatory gene programs and broad remodeling of the TME



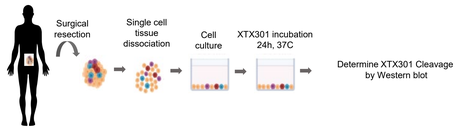
DE: differentially expressed.
Heatmap showing relative expression of top mXTX301 DE genes (N = 10 upregulated and N = 5 downregulated; by P-value). Rows (genes) were clustered according to Euclidean distance. Columns (samples) are grouped according to the treatment group (vehicle, mXTX301 [0.39 mg/kg]) and timepoint (Day 4 or Day 7). Box color tracks with z-score-transformed relative expression of each gene across samples (blue, under-expression compared to the mean; red: over-expression compared to the mean).

GO: Gene ontology; NK: natural killer cells.
Heatmap summarizing results of a pathway enrichment analysis where each box indicates the enrichment significance (-log10 Fisher *P*-value) of a gene set of interest (rows) in the Top 250 most significantly upregulated genes (by *P*-value) computed by comparing mXTX301 (0.39 mg/kg) *vs.* vehicle at Day 4 or Day 7 (columns). Color intensity tracks with significance. Gray (not found in this plot) would indicate non-significant enrichment (Fisher $P \geq 0.01$).

Figure 6: Evaluation of mXTX301 induced gene expression in the MC38 tumors by bulk RNA sequencing. (A) Comprehensive examination of global gene expression revealed a similar pattern of molecular changes for mXTX301 at Day 4 and Day 7. mXTX301 induced the upregulation of several immune-related genes such as STAT1, IL-12Rβ1, IRF1 and CXCL9. These findings were consistent with IL-12 biology. (B) mXTX301 activated immune-related transcriptional programs including IFN-γ pathway, defense response, antigen processing and presentation and adoptive immune response. mXTX301 also resulted in enrichment of signatures for T cells, NK cells, macrophages and dendritic cells in tumor.

Ex vivo proteolytic activation of XTX301 by human plasma and tumors

XTX301 cleavage by cells from human primary tumors

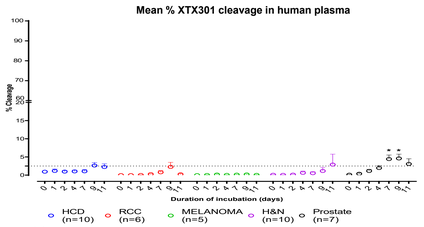


(B)

	% of samples cleaving XTX301
RCC (n=6)	83%
Colon (n=6)	83%
H&N (n=4)	75%
Prostate (n=12)	67%
Lung (n=8)	50%
Ovary (n=4)	50%
Total n=40	68%

Figure 7: *Ex vivo* cleavage analysis of XTX301 by six solid tumor types. (A) Study design: Single cell suspension was generated from human primary solid tumors. XTX301 was incubated with the cells at 37°C for 24 hrs. The cleavage was evaluated using Western Blot. (B) XTX301 was activated by cells from human primary tumors across a broad range of solid tumor types (with cleavage occurring in 50-83% of samples tested).

XTX301 was minimally cleaved by human plasma



HCD: healthy control donor
RCC: renal cell carcinoma
H&N: head & neck cancer
Data points represent mean +/- SEM of 5-10 donors

Figure 8: *Ex vivo* cleavage analysis of XTX301 by human plasma from healthy donors RCC, melanoma, prostate or H&N cancer patients. XTX301 was incubated with plasma samples at 37°C for 11 days. The cleavage was evaluated Western Blot. No significant cleavage of XTX301 was observed *ex vivo* in plasma from healthy donors, RCC, melanoma, or H&N cancer patient plasma compared to Day 0. Significant but limited cleavage of XTX301 was observed in prostate patient plasma after 7 and 9 days of incubation.

Conclusions

XTX301, a half-life extended tumor-selective IL-12 molecule, demonstrated cleavage in human tumor samples but was minimally cleaved in human plasma derived from patients with tumor burden, indicating tumor-selective activation. A mouse surrogate of XTX301, mXTX301, induced immune activation in the tumor microenvironment of a syngeneic mouse model. Furthermore, our data demonstrated that mXTX301 potently inhibited tumor growth in a mouse model with improved tolerability compared to a non-tumor selective IL-12 molecule. In conclusion, XTX301 has the potential to achieve potent anti-tumor activity while widening the potential therapeutic index of IL-12 treatment.