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Onboard, membrane-tethered IL-12 boosts potency and maintains selectivity of a Tmod NOT gate

Sara Imboden[1], Richard Lee[1], Alexandre Zampieri[1], Jon Torres[1], Alexander Kamb[1], Jingli Zhang[1]

[1]A2 Biotherapeutics, Agoura Hills, California, USA

Summary and Key Points

- Membrane-tethered IL-12 (mem-IL-12) can enhance the anti-tumor response of logic-gated, two-receptor CAR-T systems, such as Tmod™ *in vitro* and *in vivo*.
- Activation-inducible mem-IL-12 was evaluated and compared for expression, potency and safety.
- mem-IL-12 mitigates the immunosuppressive effect of TGFβ1 and boosts Tmod potency, without compromising selectivity, in a variety of *in vitro* and *in vivo* assays.

Introduction

Cytokines are involved in numerous behaviors of immune cells. Systemic administration of cytokines can initiate or enhance anti-tumor responses in some cancer patients. Unfortunately, exogenous addition of cytokines comes with a variety of challenges such as an increased risk of cytokine release syndrome (CRS). Onboard, membrane-tethered cytokines may not only mitigate the toxicity risk of exogenous cytokines but also overcome other limitations, including short half-life, and poor tissue penetration. However, potency enhancements from onboard cytokines must not compromise the therapeutic window of the engineered cells. This is especially important in products engineered with logic gates, such as the LIR-1 NOT gate, that mediates tumor-specific killing. Here, we show that membrane-tethered IL-12 (mem-IL-12) augments the potency of Tmod without impeding selectivity, in a variety of acute and long-term tumor co-culture assays and in novel *in vivo* studies.

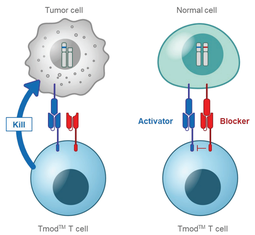

Figure 1. A schematic representation of the Tmod system [2]
Tmod, which consists of an activator and a blocker receptor, selectively kills tumor cells that express only the activator antigens, while protecting normal cells expressing both activator and blocker antigens.

Design of membrane-tethered IL-12 construct

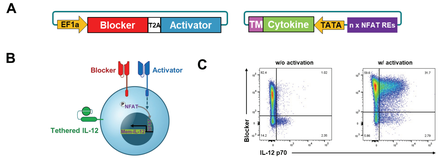

Figure 2. Co-expression of Tmod and membrane-tethered IL-12.
A & B. Tmod and mem-IL-12 are co-expressed from two separate constructs through lentiviral transduction, with one construct encoding a blocker and activator and the other construct encoding mem-IL-12. **C.** Surface IL-12 and blocker was measured by flow cytometry in T cells co-transduced with the two constructs.

mem-IL-12 is inducible & minimally shed in vitro

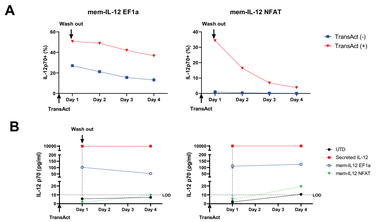

Figure 3. mem-IL-12 expression is inducible and reversible.
A. Expression of mem-IL-12 can be induced through activation with 1:100 TransAct™ overnight. **B.** Upon activation, the inducible mem-IL-12 shows low level of IL-12p70 cleavage, suggesting reduced risk for toxicity compared to constitutively expressed mem-IL-12. LOD: limit of detection

mem-IL-12 boosts Tmod potency in vitro

	Donor 1			Donor 2		
T Cell	ET50 (A)	ET50 (AB)	Selectivity Window	ET50 (A)	ET50 (AB)	Selectivity Window
MSLN CAR	0.02	0.04	2	0.03	0.04	1.3
MSLN Tmod	0.1	> 3	> 33	0.2	> 3	> 15
MSLN Tmod + mem-IL-12 NFAT	0.05	2.9	58	0.1	2.4	24

Figure 4. mem-IL-12 boosts Tmod acute *in vitro* activity, while preserving selectivity. Specific killing of MSLN Tmod with or without mem-IL-12 was measured at 96 hours with E:T ratios from 1:81 to 3:1. Tumor/A: MSLN(+)HLA-A*02(-) MS751; "Normal"/AB: MSLN(+)HLA-A*02(+) MS751.

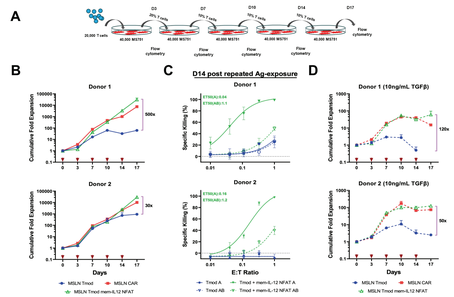

Figure 5. mem-IL-12 overcomes long-term exhaustion and suppressive tumor microenvironment. A. Schematic of *in vitro* antigen-dependent T cell expansion **B.** mem-IL-12 boosts antigen-dependent expansion of MSLN Tmod upon repeated tumor challenges and **C.** Long-term (day 14) cytotoxicity measured in E:T titrations shows selectivity is maintained by mem-IL-12 module. **D.** mem-IL-12 mitigates TGFβ1 suppression. The red arrow indicates the addition of fresh MSLN(+)A*02(-) target cells.

In vivo micro-xenograft model

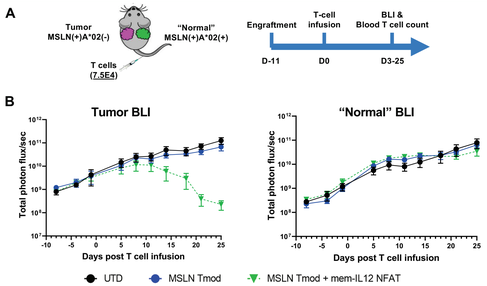

Figure 6. At the suboptimal T cell dose, mem-IL-12 boosts Tmod *in vivo* efficacy, while preserving selectivity. A. Schematic of *in vivo* dual-flank animal study. Tumor and "normal" were established by subcutaneous engraftment of 5e4 MSLN(+) HLA-A*02(-) MS751 cells (tumor; left flank) and 5e4 MSLN(+)HLA-A*02(+) MS751 cells ("normal"; right flank). 7.5e4 T cells were infused 11 days later. **B.** Tmod potency and selectivity were assessed by measuring xenograft growth via bioluminescence (BLI)

Mouse-tissue-targeting Tmod model demonstrates selectivity

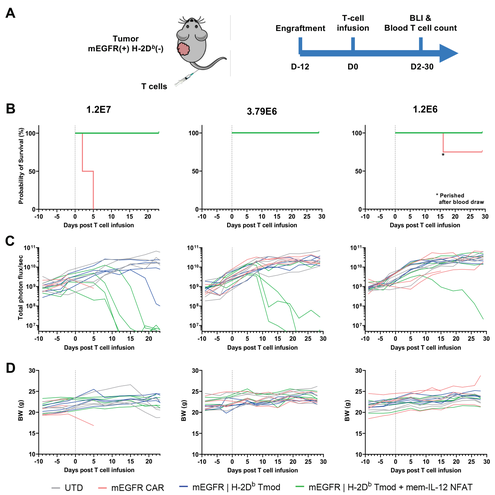

Figure 7. mem-IL-12 boosts Tmod in a surrogate mouse model.
A. Schematic of *in vivo* surrogate animal study. Tumor was established by subcutaneous engraftment of 5e4 mEGFR(+)H-2D^b(-) MS751 cells (tumor; left flank) **B.** Survival of mice infused with indicated T cell doses. Tmod potency and selectivity were assessed by **C.** the measurement of xenograft growth via bioluminescence (BLI) and **D.** animal body weight.

Modest IFNg and minimal soluble IL-12 induced by mem-IL-12 construct

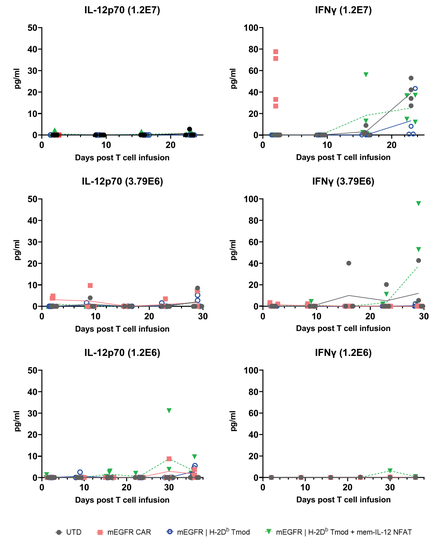

Figure 8. Low level of IL-12 cleavage and modest elevation of blood IFNγ suggests good safety profile. Though mice treated with Tmod + mem-IL-12 displayed no overt signs of toxicity caused by expression of the cytokine, we measured potential shedding of mem-IL-12, as well as its effect on secretion of IFNγ *in vivo*. Blood from the surrogate study described in Figure 7 was analyzed by CBA to measure levels of soluble IL-12 heterodimer p70 and IFNγ.

Conclusions

The findings presented here suggest that mem-IL-12 effectively enhances Tmod cytotoxicity, while preserving selectivity, and may mitigate the immunosuppressive tumor microenvironment. mem-IL-12 displayed consistent benefits to Tmod proliferation and persistence in multiple *in vitro* and *in vivo* assays. In particular, its quantitative benefits *in vivo* in two substantially different assays, micro-xenograft and surrogate Tmod models, support the hope that the mem-IL-12 booster will provide a substantial boost in dose-response of Tmod in humans, observed in the mouse models.

References

DiAndreth, B., et al., The Tmod cellular logic gate as a solution for tumor-selective immunotherapy. *Clin Immunol*, 2022.
Hamburger, A.E., et al., Engineered T cells directed at tumors with defined allelic loss. *Mol Immunol* 2020.
Oh, J., et. al. NOT gated T cells that selectively target EGFR and other widely expressed tumor antigens. *iScience*. 2024.

Visit poster #302 to hear about Tmod and signal 1 booster strategy, and poster #392 about a high-throughput screen to identify and optimize signal 1 boosters.

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www.a2bio.com jzhang@a2biotherapeutics.com