

Onboard, tethered IL-12 boosts potency of the Tmod NOT gate and preserves selectivity

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To cite: Zhang JA, Imboden S, Lee D, *et al.* Onboard, tethered IL-12 boosts potency of the Tmod NOT gate and preserves selectivity. *Journal for ImmunoTherapy of Cancer* 2025;**13**:e010976. doi:10.1136/jitc-2024-010976

► Additional supplemental material is published online only. To view, please visit the journal online (<https://doi.org/10.1136/jitc-2024-010976>).

Accepted 03 May 2025

ABSTRACT

Background To reach their full potential in cancer therapy, immune cells engineered with synthetic constructs must achieve the challenging dual objectives of potency and selectivity to overcome the key obstacle: non-specific cytotoxicity. These problems are especially challenging for solid tumor therapy, where antigen tissue specificity, accessibility, and tumor microenvironment are problematic. Cells engineered with receptors that act as synthetic logic gates promise to address the issue of tumor specificity by targeting antigen profiles rather than single antigens. Nevertheless, there are limits to the potency benefit that can be achieved at the level of the antigen-targeting receptors. One approach to enhance potency beyond the acute sensitivity of receptor activation is to co-opt a major source of ancillary stimulation in the normal immune response, cytokine receptors.

Methods Enhancing CAR-T efficacy with engineered onboard cytokines, often referred to as “armoring”, is one such approach to boost potency. However, such constructs run the risk of overriding tumor selectivity and eroding the therapeutic window. Here we design and test onboard cytokine constructs that enhance potency and preserve selectivity of a synthetic NOT logic gate construct called Tmod, potentially addressing some of the major challenges in oncology in a single synthetic design.

Results We focused especially on a module encoding membrane-tethered interleukin (IL)-12, a construct that significantly enhances Tmod antigen-dependent long-term proliferation and potency both in vitro and in vivo, without compromising the NOT gate selectivity. Notably, three substantially different in vivo models, including one that employs mouse surrogate antigens, were used to assess preclinical dose-dependent efficacy and safety. Together, these studies make a strong case for the robustness of the design.

Conclusions We conclude that the mem-IL-12 module can be combined with multiple Tmod constructs to boost efficacy and persistence while preserving the on-tumor selectivity.

BACKGROUND

Cell therapy has the power to cure patients with cancer by specifically targeting their tumors with immune cells designed to seek out and eliminate malignant cells in the

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Two things are clear from the results of cell therapy trials in solid tumors: (1) tumor versus normal tissue selectivity is often problematic; and (2) in the cases where therapies are tolerated, efficacy is much less impressive than in blood cancer cell therapy. To address the second problem, a wide variety of methods to enhance adoptive transfer of T cells with cytokines, including exogenous administration and engineered cytokine expression modules, have been tested preclinically and in the clinic. Only a handful of studies have addressed both problems simultaneously, placing a premium on efforts to do so.

WHAT THIS STUDY ADDS

⇒ The work presented here demonstrates that a robust NOT gate (Tmod) designed to enforce selective killing of tumor cells can be enhanced with membrane-tethered cytokines, notably interleukin-12. Critically, these enhancements do not compromise the selectivity of the Tmod mechanism.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The results are important because they illuminate a path toward using logic gates, Tmod in particular, in solid tumors where the twofold problem of tumor selectivity and potency remains a major impediment.



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body. Compared with traditional therapeutics such as antibodies and small molecules, cells are complex machines that do more than simply bind target molecules. They engage in sophisticated behaviors such as integration of multiple input signals, replication, and migration. In principle, a therapy that uses the inherent complex functionality of the immune system can exploit many of the features that evolved to combat pathogenic organisms, including the amplification of the response from a single immune cell that recognizes abnormal cells.

A promising new subtype of engineered immune cells involves synthetic logic gates;

that is, receptor systems designed to recognize and respond to complex molecular patterns on target cells. By analogy with Boolean logic used in computer designs, examples of synthetic biology logic gates include OR, AND, and NOT gates that respond to different combinations of antigen pairs (see for reviews^{1,2}). For example, a NOT gate is a type of cellular control system that activates when two conditions are satisfied by a target cell it encounters: (1) the target cell expresses an activating or A-antigen; and (2) the target cell lacks expression of a second blocking or B-antigen. This molecular device, exemplified by the Tmod dual-receptor system, can exploit situations where healthy normal cells express both the A- and B-antigens and cancer cells express only the A-antigen and not the B-antigen. Such situations occur, for example, when cancer cells have lost expression of one allele in a phenomenon known as loss of heterozygosity (LOH). Tmod constructs where tumor selectivity is guided by the HLA-A*02 antigen expressed on normal cells are in the clinic³ (NCT05736731; NCT06051695). Tmod can also be used in situations that do not involve genetic deletions; for example, in certain blood cancers where malignant cells lack expression of a B-antigen due to epigenetic effects.^{4,5}

Although cell therapy has achieved dramatic success in several blood cancer settings (eg, non-Hodgkin's lymphoma and multiple myeloma), solid tumor cell therapy has proved more challenging. Part of the challenge is the scarcity of truly tumor-specific antigens. Although Tmod provides a potential solution to the problem of specificity, solid tumors present additional obstacles. For example, engineered cells may not readily encounter antigens expressed on tumors outside the blood vessels and must migrate across vessel walls to exert their effector function in the suppressive tumor microenvironment. These constraints place a premium on the potency of the engineered immune cells and on methods to enhance such potency. However, in the case of engineered logic gates such as Tmod, enhancement must not override the selectivity achieved by the logic gate. It is not obvious how such a benefit can be implemented, given the complex nature of signal integration and crosstalk in immune cells (eg, reviewed in Adelaja and Hoffmann).⁶

The majority of efforts to boost potency rely on stimulation of the principal signaling receptors that operate in the adaptive immune response, namely antigen receptors (signal 1), co-stimulatory receptors (signal 2), and cytokine receptors (signal 3).⁷ Although simple to conceive in principle, the behavior of these complex systems is far from predictable. Among the challenges of engineering such systems and/or providing selective boosts to immune cell sensitivity are the following: (1) systemic toxicity of agents such as co-stimulatory ligands and cytokines^{8,9}; (2) short half-life of many natural immunostimulants in the circulation; (3) poor tissue penetration of peptides and proteins, including cytokines; and (4) the risk of promoting immune cell exhaustion via hyperstimulation.

In the studies described here, signal 3 is shown to provide Tmod with benefits expected of an amplifier of the adaptive immune response. Large-effect cytokines (interleukin (IL)-2, IL-7, IL-12, IL-15, IL-18, IL-21) were tested exogenously in the context of the Tmod NOT gate. All these cytokines enhanced T-cell activity in at least one of two Tmod constructs, CEA | HLA-A*02 and/or MSLN | HLA-A*02, without overriding the HLA-A*02 blocker, a key requirement of booster function in the context of Tmod. We focused on membrane-tethered IL-12 (mem-IL-12) and showed that mem-IL-12 boosted antigen-dependent activity of Tmod constructs by $\geq 10\times$ in a variety of longer-term in vitro and in vivo assays without compromising the selectivity window of Tmod, and also mitigated the immunosuppressive effect of TGF- β (transforming growth factor- β), thought to be a key component of the tumor microenvironment. Moreover, minimal soluble IL-12 was observed both in vitro and in the blood of treated mice, consistent with an acceptable safety profile of a Tmod product that carries mem-IL-12.

RESULTS

Optimal activation and proliferation of Tmod requires signal 3

In an initial series of experiments, we confirmed that Tmod cells display the same requirements as unmodified T cells for cytokine signaling to achieve maximal antigen-dependent responses in vitro.¹⁰ We used IL-2 to represent large-effect common gamma chain cytokines.¹¹ We tested Tmod constructs with CARs (chimeric antigen receptors) directed at either CEA (carcinoembryonic antigen) or MSLN (mesothelin) tumor-associated antigen and a blocker directed at the HLA-A*02 antigen (figure 1A, online supplemental figure S1A). Activator and blocker were encoded as part of a two-gene single transcript with a “self-cleaving” 2A peptide (T2A) that generates the two independent receptors.¹² One type of construct (Tmod(sh)) encoded a β_2 microglobulin (B2M)-directed shRNA to reduce cis-binding of the blocker to endogenous HLA-A*02 molecules in autologous T cells (online supplemental figure S1A,B). Both CEA and MSLN Tmod(sh) constructs are currently undergoing early clinical studies in HLA-A*02 heterozygous patients whose tumors have clonal loss of the HLA-A*02 allele (LOH; NCT05736731; NCT06051695).

CAR, Tmod, Tmod(sh), and untransduced (UTD) T cells were cultured for 5 days under indicated growth conditions and monitored for expansion (figure 1B). In the absence of IL-2 and CD3/CD28 beads (TransAct), CAR-T, Tmod, Tmod(sh) and UTD all died off. In the presence of IL-2 (300 IU/mL) T cells expanded twofold to threefold. Addition of TransAct, a simultaneous signal 1 and 2 stimulus, increased proliferation ~ 3 -fold above IL-2 alone, peaking at ~ 5 – 7 -fold expansion relative to the starting cell number. TransAct alone (ie, without IL-2) temporarily promoted T-cell expansion, but ultimately was unable to match the combined effect of both agents

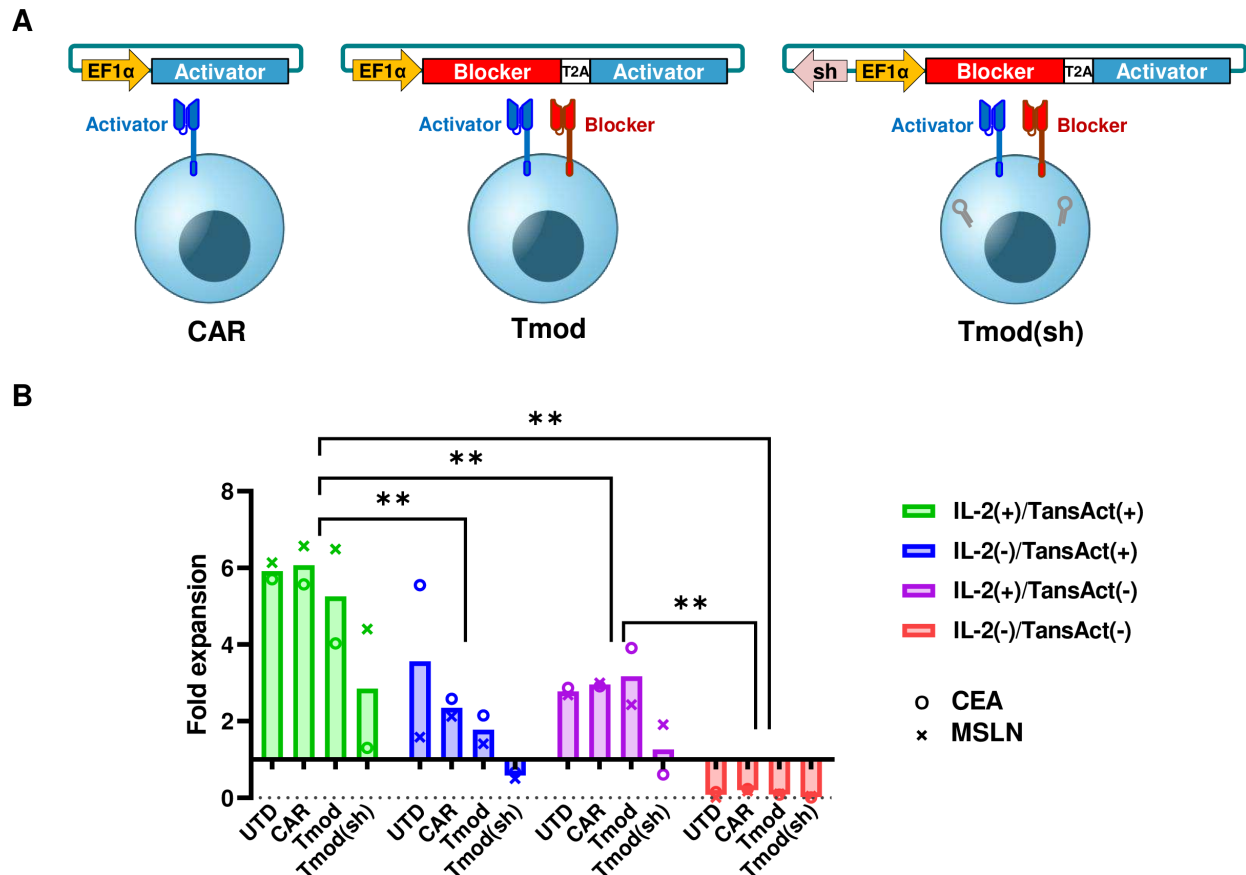


Figure 1 Engineered T cells respond best to multiple signals. T cells transduced with constructs encoding CAR, Tmod, or Tmod(sh) (A) from an HLA-A*02(-) donor (Donor 1) were placed under four different growth conditions as indicated for 5 days and monitored for fold expansion. (B) Either CEA | HLA-A*02 constructs (○) or MSLN | HLA-A*02 constructs (×) and corresponding controls were used. P values were calculated using paired t-tests. **p<0.01. CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; IL, interleukin; MSLN, mesothelin; UTD, untransduced.

(TransAct+IL-2) over the longer term. The combined data are aligned with a large body of evidence about the roles of signal 1, 2 and 3 in T-cell biology and demonstrated the value of a signal 3 stimulus in the context of Tmod.

Exogenous cytokines boost activity without overriding the Tmod blocker

To further assess the effects of cytokines on Tmod, we expanded the number of exogenous cytokines tested in vitro and examined the potency and selectivity of boosted Tmod cells. The cytokine panel included not only common gamma chain cytokines but also IL-12¹³ and IL-18.¹⁴ In a first series of experiments, we focused on the CEA | HLA-A*02 Tmod construct. HLA-A*02(-) primary T cells were transduced with the CEA Tmod construct and co-cultured with either CEA(+)HLA-A*02(-) target cells (Target A cells) that model tumor or CEA(+)HLA-A*02(+) target cells that model normal cells (Target AB cells; see online supplemental methods). These co-cultures were treated with different doses of exogenous cytokines. All cytokines displayed a dose-response for boosted cytotoxicity against Target A cells (tumor) (except for IL-12, which appeared to elicit Tmod maximum response at as low as 0.04 ng/mL) (figure 2A). However, none of the cytokines tested

boosted killing of Target AB cells (“normal”) by CEA Tmod cells to a high degree, suggesting that the cytokines explored here boost Tmod on-target cytotoxicity without overriding the blocker.

The effect of these cytokines on acute antigen-dependent proliferation was tested in similar 4-day co-cultures using CellTrace Violet dilution versus time as the readout. Flow cytometry revealed that most cytokines added at response-saturating concentrations stimulated proliferation of CEA Tmod in the presence of Target A cells (tumor), but not Target AB cells (“normal”) (except for IL-2 and IL-15, which also moderately promoted antigen-independent Tmod proliferation) (figure 2B). These results were confirmed with an alternative proliferation tracker, CellTrace CFSE (carboxyfluorescein succinimidyl ester; online supplemental figure S2A). Lastly, these exogenous cytokines not only boosted expansion but also stimulated production of other proinflammatory cytokines, such as interferon (IFN)-γ and tumor necrosis factor, in an antigen-dependent fashion (except for IL-15, which also slightly increased IFN-γ production with “normal” targets) (online supplemental figure S2B,C). Thus, most of the tested

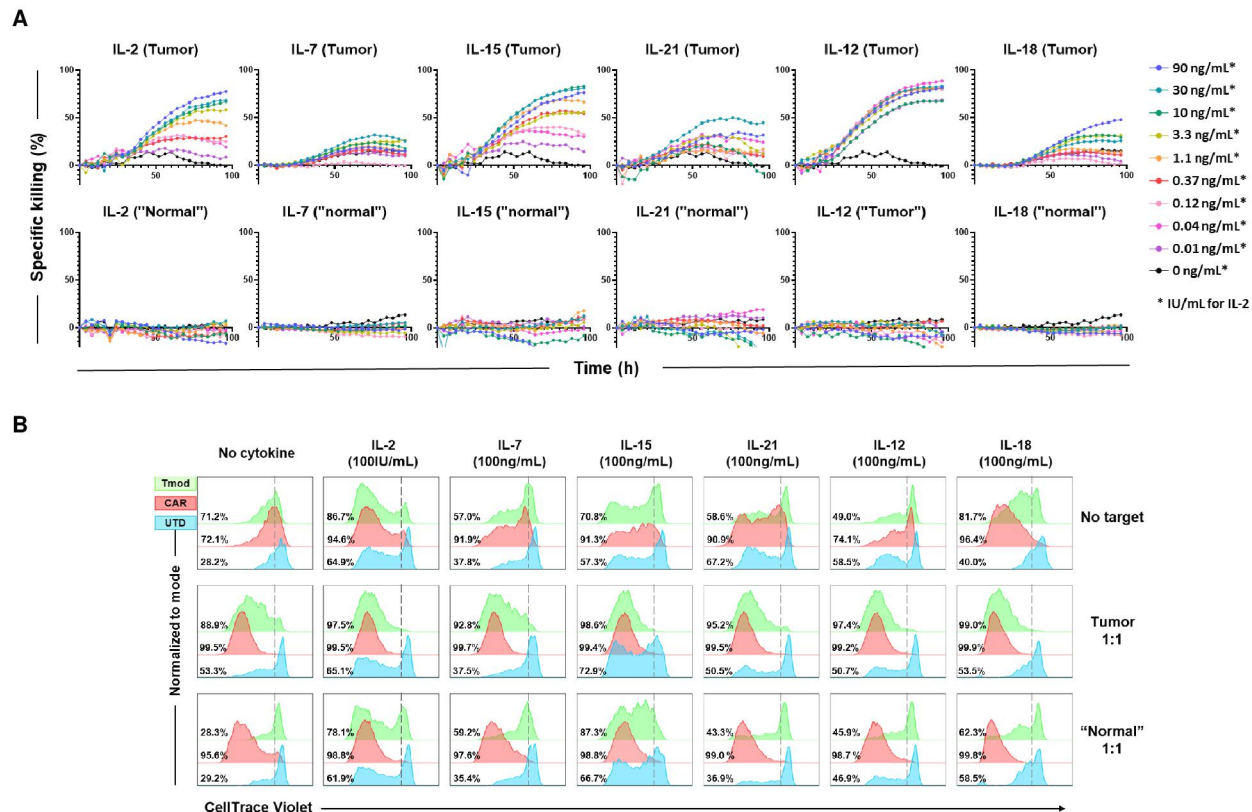


Figure 2 Exogenous cytokines boost Tmod cytotoxicity and antigen-dependent proliferation. (A) T cells were transduced with a Tmod construct encoding a CEA activator and HLA-A*02 blocker and maintained in 300 IU/mL IL-2 prior to assay. CEA Tmod or UTD cells were co-cultured with target cells at E:T=1:1 for 96 hours. Exogenous cytokines at indicated concentration (all concentration units in ng/mL except for IL-2 in IU/mL) were added in both Tmod and UTD wells at time=0. To account for non-specific killing induced by allogeneic reaction, which could be enhanced by exogenous cytokines, the percentage of specific killing was calculated as the difference of live targets between Tmod wells versus corresponding UTD T-cell wells, then normalized to live targets of the corresponding UTD T-cell wells (see online supplemental methods). $n=4$ replicates per sample. Error bars are not shown. Tumor: CEA(+)HLA-A*02(−) NCI-H508; "normal": CEA(+)HLA-A*02(+) NCI-H508. (B) CellTrace Violet-treated UTD, CEA CAR-T, or CEA Tmod cells were co-cultured either with tumor target cells (CEA(+)HLA-A*02(−) H508) or "normal" target cells (CEA(+)HLA-A*02(+) H508), at E:T=1:1 for 4 days. Exogenous cytokines were added on day 0. On day 4, T-cell proliferation was measured based on CellTrace Violet dilution (ie, cells to the left of the dotted line underwent at least one cell division). Cells were gated on CD3(+) (ie, UTD), CD3(+)/CAR(+) (ie, CEA CAR-T), or CD3(+)/blocker(+) (ie, CEA Tmod). The percentage of proliferated cells is indicated in the flow plot. HLA-A*02(−) donor (Donor 2). CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; E:T, effector cells to target cells; IL, interleukin; UTD, untransduced.

cytokines boosted Tmod potency without compromising the selectivity of the Tmod blocker mechanism.

Onboard cytokines boost acute, selective cytotoxicity of Tmod in vitro

Based on these experiments with exogenous cytokines, efforts were undertaken to construct onboard variants of several large-effect cytokines. We focused particularly on IL-12, due to its relatively high sensitivity in the context of Tmod. Two types of membrane-tethered constructs were designed (figure 3A): one driven by a constitutively activated promoter (EF1 α) and one driven by a minimal TATA promoter regulated by multiple NFAT (nuclear factor of activated T cells) response elements (4 \times NFAT or 6 \times NFAT; see online supplemental methods).^{15–18} Secreted IL-12 driven by the EF1 α promoter was cloned as a control to evaluate whether membrane-tethered IL-12 (mem-IL-12) functioned at a level equivalent to

soluble IL-12. Surface expression was confirmed for the mem-IL-12 constructs, and the regulation of the NFAT promoters was demonstrated by TransAct activation that increased surface expression of IL-12 (figure 3B; online supplemental figure S3A).

The mem-IL-12 EF1 α construct was compared with the secreted IL-12 EF1 α construct in vitro with respect to enhancement of antigen-dependent CEA Tmod (with a B2M-directed shRNA) activity (figure 3C; online supplemental figure S3B). ET50 values calculated from E:T (effector cells to target cells) ratio titrations (see online supplemental methods) were used to estimate acute effects on potency and selectivity. Both onboard IL-12 constructs boosted acute killing of Target A cells (tumor) by CEA Tmod $\sim 8\times$ with little negative effect on blocking; that is, CEA Tmod cells largely spared Target AB cells ("normal") at an E:T ratio where Target A cells were killed,

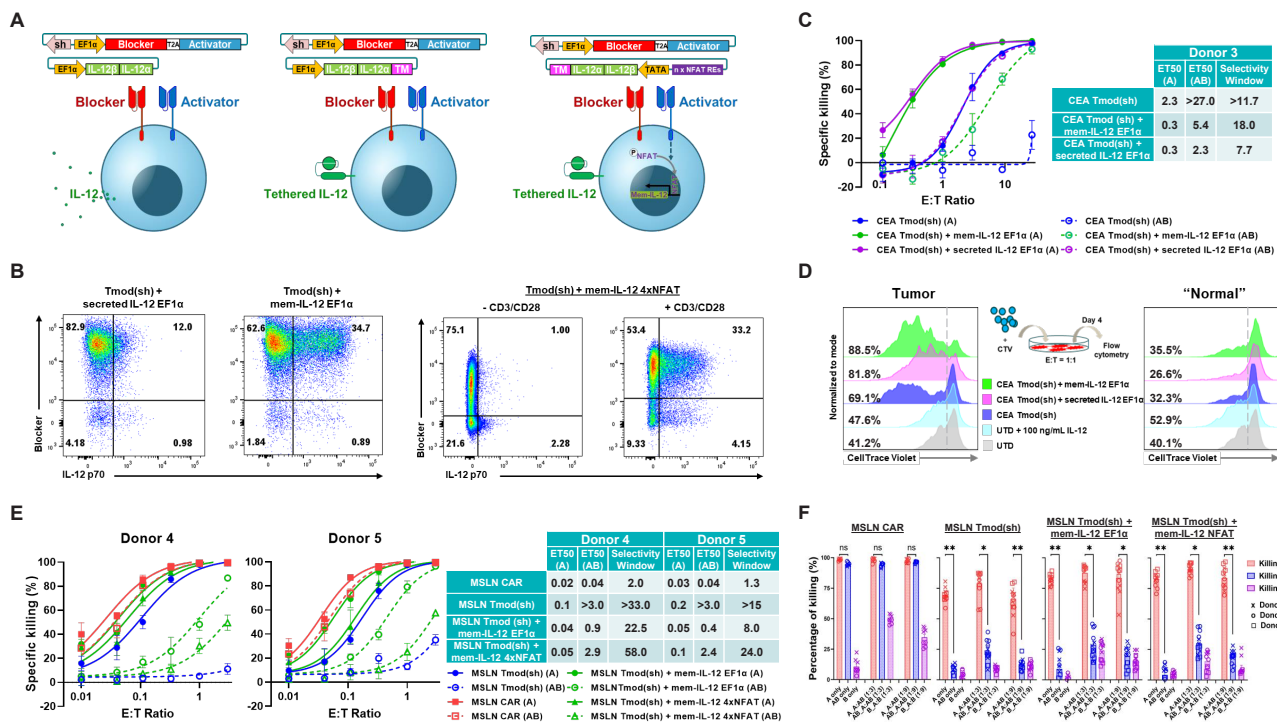


Figure 3 mem-IL-12 boosts Tmod(sh) T-cell activity, while preserving selectivity. (A) Tmod(sh) and secreted IL-12 or membrane-tethered IL-12 are co-expressed from two separate lentiviral constructs, with one construct encoding HLA-A*02 blocker and activator and the other construct encoding IL-12 heterodimer either alone (secreted) or linked to the B7 transmembrane domain (membrane-tethered). The expression of mem-IL-12 is either constitutively regulated by the EF1 α promoter, or activation-inducible regulated by four or six NFAT response elements (n \times NFAT REs). (B) mem-IL-12 expression was determined by flow cytometry using anti-IL-12 p70. Two-virus transduction yielded 20–40% of CEA or MSLN Tmod(sh) cells coexpressing mem-IL-12. See online supplemental figures for more data. (C) Acute specific cytotoxicity of CEA Tmod(sh) was enhanced by constitutively expressed mem-IL-12 or secreted IL-12. CEA Tmod(sh) with or without onboard IL-12 were co-cultured with NCI-H508 targets for 48 hours with E:T ratios from 1:9 to 27:1. The percentage of specific killing was calculated as the difference of live targets between Tmod wells versus corresponding UTD T-cell wells, then normalized to the corresponding UTD T-cell wells (see online supplemental methods). n=4 replicates per sample. Error bars represent SD. E:T versus killing curves were fit by a 4-parameter non-linear regression analysis. The ET50s were interpolated from fitted curves, and selectivity windows were calculated as ET50 on “normal” divided by ET50 on tumor. (D) Antigen-induced proliferation of CEA Tmod(sh) cells was enhanced by constitutively expressed mem-IL-12 or secreted IL-12 after 4 days of co-culture. On day 4, T-cell proliferation was measured based on CellTrace Violet dilution (ie, cells to the left of the dotted line underwent at least one cell division). Cells were gated on CD3(+) (ie, UTD) or CD3(+)blocker(+) (ie, CEA Tmod(sh) or CEA Tmod(sh) + mem-IL-12). The percentage of proliferated cells is indicated in the flow plot. Tumor or A: CEA(+)HLA-A*02(–) NCI-H508; “normal” or AB: CEA(+)HLA-A*02(+)NCI-H508. An HLA-A*02(–) donor (Donor 3) was tested in (C) and (D). (E) Acute specific cytotoxicity of MSLN Tmod(sh) was enhanced by either constitutively expressed (EF1 α) or inducible (4 \times NFAT) mem-IL-12. MSLN Tmod(sh) with or without mem-IL-12 was co-cultured with MS751 targets for 96 hours with E:T ratios from 1:81 to 3:1. The specific killing was analyzed similarly as in (C). n=4 replicates per sample. Error bars represent SD. Tumor or A: MSLN(+)HLA-A*02(–) MS751; “normal” or AB: MSLN(+)HLA-A*02(+) MS751. Two HLA-A*02(+) donors (Donor 4 and 5) were tested. (F) Tumor selectivity of Tmod(sh) in tumor and “normal” mixed co-cultures. MSLN(+)HLA-A*02(–)jffLuc(+) HeLa (tumor or A) were mixed with MSLN(+) HLA-A*02(+)jffLuc(+) HeLa (antigen-positive “normal” or AB), or MSLN(–)HLA-A*02(–)jffLuc(+) HeLa (antigen-negative “normal” or B) at 1:3 or 1:9 ratios of tumor to “normal” cells, and co-cultured with either UTD or MSLN-targeting effector T cells at E:T=1:2. After 48 hours of co-culture, luciferase assays were performed to determine the percentage of killing compared with UTD controls (see online supplemental methods). Low levels of non-specific killing of normal targets by activated CAR-T or Tmod(sh) (with or without mem-IL-12) were observed in mixed co-culture conditions (AB_A:AB or B_A:B), likely due to enhanced bystander killing (eg, allogeneic reaction boosted by increased interferon- γ). Three HLA-A*02(+) donors were tested, two (Donor 4 and 5) for CAR-T and three (Donor 4, 5, and 6) for MSLN Tmod(sh) + mem-IL-12. n=4 replicates per sample. Error bars represent SD. P values were calculated using paired t-tests. *p<0.05; **p<0.01; ns: not significant. CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; E:T, effector cells to target cells; IL, interleukin; MSLN, mesothelin; NFAT, nuclear factor of activated T cell; REs, response elements; UTD, untransduced.

suggesting mem-IL-12 was roughly similar to soluble IL-12 as a booster. The acute effect of mem-IL-12 manifested as a left shift in the selectivity window; that is, an increase in the potency while maintaining the ratio between ET50s of cytotoxicity against tumor and “normal” cells. Furthermore, by restricting expression to the T-cell surface, mem-IL-12 is less likely to cause IL-12-related systemic toxicity in vivo (discussed in later sections). Short-term proliferation correlated with cytotoxicity; that is, mem-IL-12 promoted measurable proliferation when Tmod cells were exposed to Target A cells (tumor), but not when exposed to Target AB cells (“normal”) (figure 3D).

To extend these results, we tested acute behavior of both EF1 α -driven mem-IL-12 and 4 \times NFAT-driven inducible mem-IL-12 with a second Tmod construct, MSLN | HLA-A*02 Tmod (with a B2M-directed shRNA). This confirmatory series of experiments used a parallel set of isogenic target cell lines based on MS751 instead of NCI-H508.¹² With MSLN Tmod, both booster modules produced smaller, but reproducible, effects on ET50 and did not compromise selectivity (figure 3E). Additionally, MSLN Tmod cells with either booster module maintained the robust selectivity for tumor cell killing when incubated with mixed cultures of tumor and “normal” cells (figure 3F). In contrast, MSLN CAR-Ts killed both MSLN(+) tumor and “normal” target cells indiscriminately.

Due to the two-vector, two-step transduction process (see online supplemental methods), the percentage of blocker and mem-IL-12 double-positive cells ranged from 20% to 40% for both mem-IL-12 constructs, measured by flow cytometry. However, despite the percentage variation of Tmod(+)mem-IL-12(+) cells, we did not observe significant differences among products in terms of potency enhancement and selectivity (data not shown), suggesting mem-IL-12 may act both in cis and in trans with other T cells in close proximity.

Finally, we compared mem-IL-12 to other membrane-bound signal 3 boosters. One of these, a dimerizing version of IL-7R α (di-IL7R α) has been shown to stimulate the growth of CAR-T cells in which it was expressed.¹⁹ A series of similar assays demonstrated di-IL7R α slightly enhanced cytotoxicity of CEA or MSLN Tmod in vitro, while maintaining selectivity (online supplemental figure S3C–G). Two other constructs, membrane-tethered IL-2 (mem-IL-2) and IL-18 (mem-IL-18), were tested for acute effects on MSLN Tmod after confirming surface expression. While mem-IL-2 behaved inconsistently in terms of expression and function (data not shown), constitutively expressed mem-IL-18 reproducibly boosted potency without disrupting blocker function (online supplemental figure S3H,I). The effects of IL-18 and di-IL7R α on the acute selectivity window were similar to mem-IL-12 with regard to left-shifting the dose-response measured by E:T ratio titration. However, none of these other membrane-bound signal 3 boosters exceeded the acute potency enhancement of mem-IL-12.

Onboard cytokines provide a long-term boost in vitro

We next explored the effect of mem-IL-12 on the long-term behavior of MSLN Tmod cells (figure 4A). Both mem-IL-12 constructs (EF1 α and NFAT) enhanced antigen-dependent expansion over time in assays where target cells were replenished every 3–4 days (figure 4B). Importantly, the growth-inhibitory effect of high-concentration TGF- β 1 (10 ng/mL) was substantially overcome by Tmod cells that also expressed mem-IL-12 (figure 4C). Furthermore, even after four to five rounds of tumor rechallenge, the majority of Tmod(+) cells (including both Tmod (+)mem-IL-12(+) and Tmod(+)mem-IL-12(-)) with either booster module expressed PD-1 (programmed cell death protein 1) at a lower level compared with unboosted Tmod control and CAR-T, and retained their potency and selectivity against target cells (figure 4D,E). ET50 values and selectivity window were similar after 14 days in serial co-culture with target cells compared with the assay start (see figure 3E). These results suggest that mem-IL-12 boosts Tmod antigen-dependent proliferation over time without sacrificing potency and selectivity or causing exhaustion.

A subset of similar long-term assays was conducted with mem-IL-18 and di-IL7R α (online supplemental figure S4). Results were generally comparable to those of mem-IL-12, though there were certain differences. In particular, the di-IL7R α construct tested with MSLN Tmod failed to confer resistance to TGF- β inhibition (online supplemental figure S4B). As in the acute assays, neither mem-IL-18 nor di-IL7R α was superior to mem-IL-12 in any of the long-term in vitro behaviors tested in Tmod.

mem-IL-12 boosts Tmod cytotoxicity and antigen-dependent expansion in vivo

We next tested the potency and selectivity of mem-IL-12 constructs in vivo with MSLN Tmod (with a B2M-directed shRNA). The in vivo model consisted of NSG mice bearing two micro-xenografts, one on each flank. The micrografts were composed of the same MS751 isogenic cell lines used to test selectivity in vitro; namely, MSLN(+)HLA-A*02(+) MS751 cells representing “normal” cells on the left flank and isogenic MS751 MSLN(+)HLA-A*02(-) MS751 cells representing tumor cells on the right flank. MSLN constructs were characterized prior to infusion into mice with respect to surface expression of receptors and mem-IL-12, as well as functional activity (online supplemental figure S5A; figure 3E). 11–12 days after injection of 50,000 graft cells into each flank, the mice were infused via tail vein injection with three different doses of MSLN Tmod cells spanning a 100-fold range. UTD T cells were included for comparison, and bioluminescence imaging (BLI) and body weights were monitored over time (figure 5A,B; online supplemental figure S5B). At the high dose (7.5e6

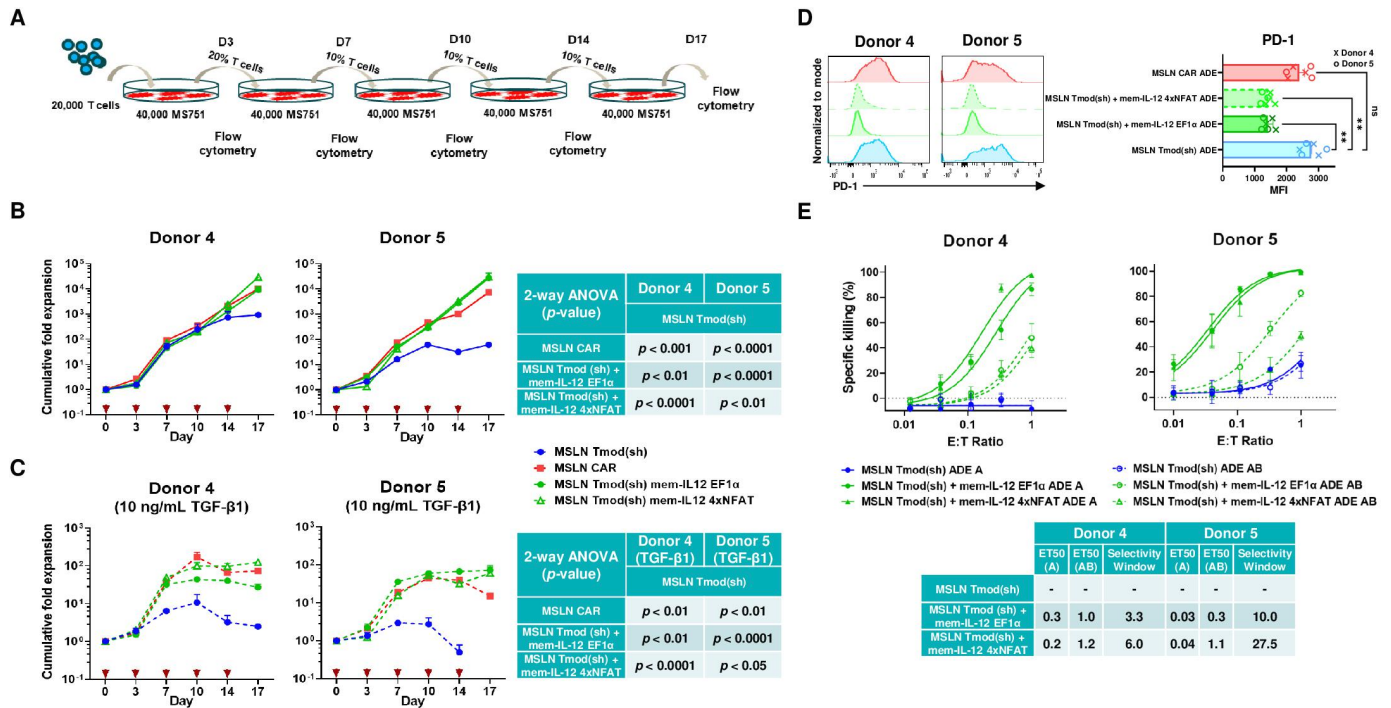


Figure 4 mem-IL-12 boosts antigen-dependent expansion of MSLN Tmod(sh) in the presence of TGF- β . (A) Experimental workflow. (B) Expansion versus time. Both constitutively expressed (EF1 α promoter) and activation-induced (NFAT promoter) mem-IL-12 enhance antigen-dependent expansion of MSLN Tmod(sh) cells on repeated tumor exposure and (C) partially overcome TGF- β -mediated inhibition, which was added fresh with every round of new targets. Tumor target: MSLN(+) HLA-A*02(-) MS751. n=2 replicates per sample. P values were calculated using two-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (D) and (E) mem-IL-12 boosts MSLN Tmod(sh) cells in long-term potency assays while maintaining selectivity. CAR(+) or blocker(+) T cells from the experiment shown in (B) were collected after 14~18 days of repeated tumor exposure, then (D) measured for surface PD-1 expression and (E) specific cytotoxicity and selectivity. PD-1 flow plot is a representative of n=3 repeats per donor. Cells were gated on CD3(+)CAR(+) (ie, CAR-T) or CD3(+)blocker(+) (ie, Tmod(sh) or Tmod(sh) + mem-IL-12). Error bars represent SD. P values were calculated using t-tests. ** $p < 0.01$; ns: not significant. The specific killing was analyzed similarly as in figure 3C. n=4 replicates per sample. Error bars represent SD. Donors tested here were the same donors tested in figure 3E. ADE, antigen-dependent expansion; ANOVA, analysis of variance; CAR, chimeric antigen receptor; E:T, effector cells to target cells; IL, interleukin; MSLN, mesothelin; NFAT, nuclear factor of activated T cell; PD-1, programmed cell death protein 1; TGF- β , transforming growth factor- β ;

cells/mouse), unboosted Tmod displayed no selectivity and cleared both the tumor and “normal” grafts, with the kinetics of killing possibly more rapid for the tumor grafts. MSLN Tmod+mem-IL-12 (4xNFAT or EF1 α) constructs were not tested at the top dose, but at the middle dose (7.5e5), these constructs followed the non-selective behavior of unboosted MSLN Tmod at the high dose (7.5e6); that is, they killed both grafts indiscriminately. In contrast, at this middle dose, unboosted MSLN Tmod was selective (ie, the blocker did not override the activator), but with slightly reduced tumor clearance kinetics compared with the boosted versions. At the low dose (7.5e4), only the mem-IL-12-boosted constructs killed the tumor graft; at this dose, the “normal” graft was spared. Together, these data indicated that in this in vivo model, MSLN Tmod displays a ~10 \times selectivity window with respect to dose-response, whether in boosted or unboosted format. Thus, our findings suggest that mem-IL-12 induces a left-shifted dose-response, both on-target and off-target, and enables

effective tumor clearance and selectivity at a ~10 \times lower dose (ie, 7.5e4) (figure 5B), in line with the in vitro selectivity displayed in E:T titration experiments (figure 3E).

Other measurements of the mice were also consistent with in vitro studies. There was no negative effect on body weight, suggesting a highly specific reaction to MSLN and minimal off-target effect (online supplemental figure S5B). Compared with the unboosted Tmod, blood Tmod cell counts were elevated in mice treated with Tmod+mem-IL-12 cells (figure 5C). In addition, EF1 α -treated and NFAT-mem-IL-12-treated mice had modestly increased levels of IFN- γ in blood, though the EF1 α construct produced more than the NFAT construct, particularly in the middle dose (7.5e5) group (figure 5D). Both effector T-cell counts and IFN- γ levels suggested that part of the efficacy boost of mem-IL-12 is due to increased proliferation and IFN- γ secretion. Together, these results were consistent with an efficacy boost of at least 10 \times induced by mem-IL-12, while preserving a selectivity window.

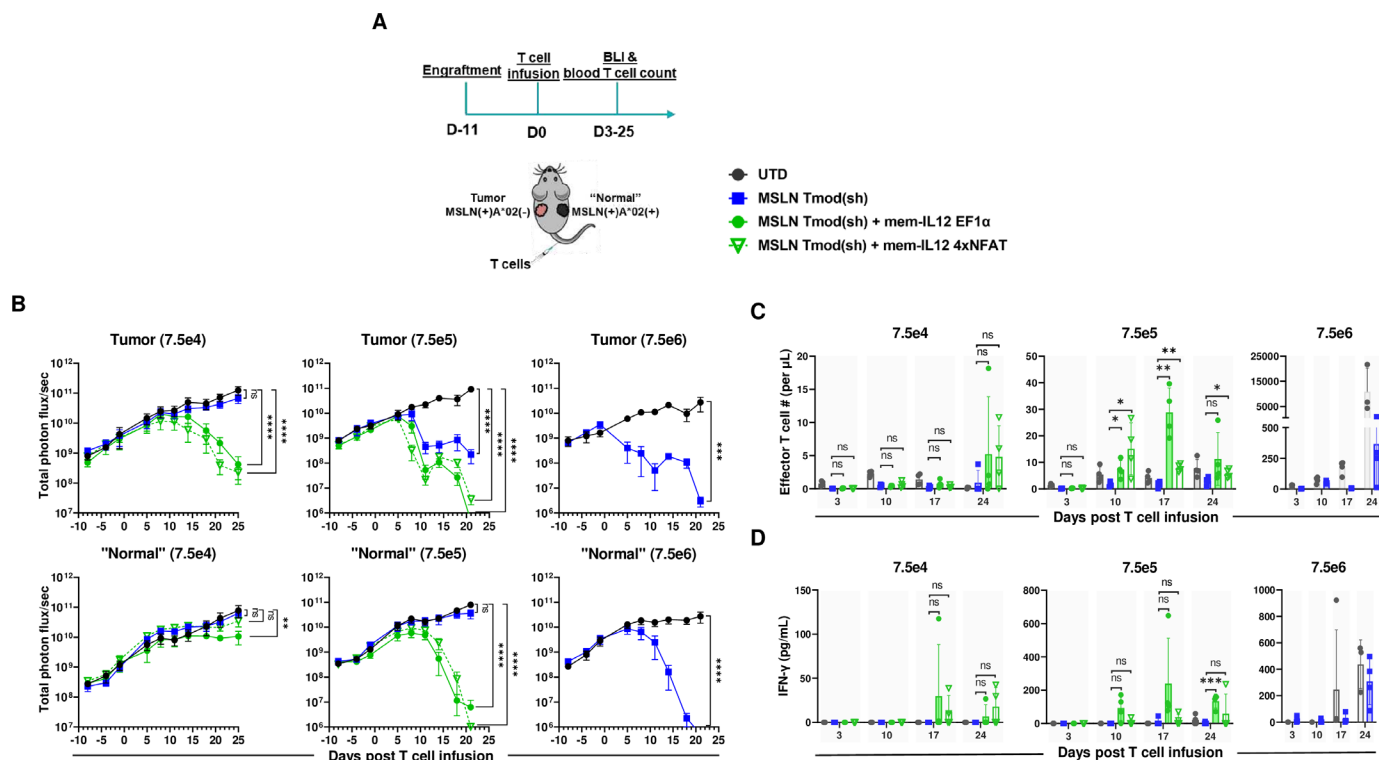


Figure 5 mem-IL-12 boosts Tmod in vivo activity and expansion, while preserving selectivity. (A) Schematic of dual-flank animal study. Tumor and "normal" were established by subcutaneous engraftment of 5e4 MSLN(+)/HLA-A*02(-) MS751 cells (tumor; left flank) and 5e4 MSLN(+)/HLA-A*02(+) MS751 cells ("normal"; right flank), and 7.5e4, 7.5e5, or 7.5e6 transduced T cells were infused 11 days later. n=4 mice per cohort. Donor 4 was tested here. (B) Tmod potency and selectivity were assessed based on xenograft growth via bioluminescence imaging (BLI). Error bars represent SEM. P values for BLI changes of Tmod(sh) groups against UTD group were calculated using two-way analysis of variance. **p<0.01; ***p<0.001; ****p<0.0001; ns: not significant. At the 7.5e4 dose, both constitutively expressed and inducible mem-IL-12 enhanced MSLN Tmod(sh) in vivo tumor clearance and expansion by ~10×, while maintaining a ~10× selectivity window. Blood was collected to measure T-cell expansion (C) and serum IFN-γ level (D). Effector T cells were counted using flow cytometry by gating for hCD3(+) (ie, UTD) or hCD3(+)blocker(+) (ie, Tmod(sh) or Tmod(sh) + mem-IL-12) in blood. IFN-γ was measured using Cytometric Bead Array (see online supplemental methods). Effective T-cell (transduced) doses are listed above the graphs. P values for mem-IL-12 boosted Tmod against Tmod at different time points (except for the pairs in which all samples had IFN-γ below the limit of detection at 10 ng/mL) were calculated using t-tests. *p<0.05; **p<0.01; ***p<0.001; ns: not significant. Error bars represent SD. Large elevations in blood T-cell counts and serum IFN-γ levels were observed 14–21 days post T-cell infusion in both 7.5e5 groups, suggesting significant xenogeneic reactions. IFN, interferon; IL, interleukin; MSLN, mesothelin; NFAT, nuclear factor of activated T cell; UTD, untransduced.

A similar set of experiments was conducted using MSLN Tmod±di-IL7Rα. Though the constructs displayed a clear response, the efficacy benefit observed with the di-IL7Rα booster was significantly smaller than with mem-IL-12 (online supplemental figure S5C,D), accompanied by minimal increase in blood IFN-γ and cell number at early time points (in contrast to mem-IL-12 constructs which showed a substantial increase in cell number) (online supplemental figure S5E).

To test whether the activity of mem-IL-12 and di-IL7Rα was antigen-dependent in vivo, we infused T cells bearing either constitutively expressed mem-IL-12 or di-IL7Rα modules into NSG mice without xenografts. Neither weight loss nor enhanced T-cell proliferation was observed in mouse blood through day 16, though both T-cell numbers and IFN-γ levels began to rise by day 22 (online supplemental figure F–H). This late increase was likely due to a xenogeneic response, as it was also observed in

the UTD control mice, though enhanced by the boosters, particularly mem-IL-12. These results confirmed observations from the in vitro studies (figures 2 and 3D; online supplemental figure S2) and support the conclusion that the effect of mem-IL-12 (and di-IL7Rα) is antigen-dependent, as they require the presence of an antigen-expressing xenograft.

In vivo confirmation of mem-IL-12 enhancement in a surrogate mEGFR model

Having confirmed the effects of mem-IL-12 on potency and selectivity using in vivo xenografts, we next tested the influence of NFAT-controlled inducible mem-IL-12 on a Tmod construct that targets murine antigens; specifically mEGFR and H-2D^b, a murine major histocompatibility complex class I paralog expressed in all nucleated cells. The in vivo model consisted of NSG mice bearing a single-flank micro-xenograft of mEGFR(+)/H-2D^b(-) MS751

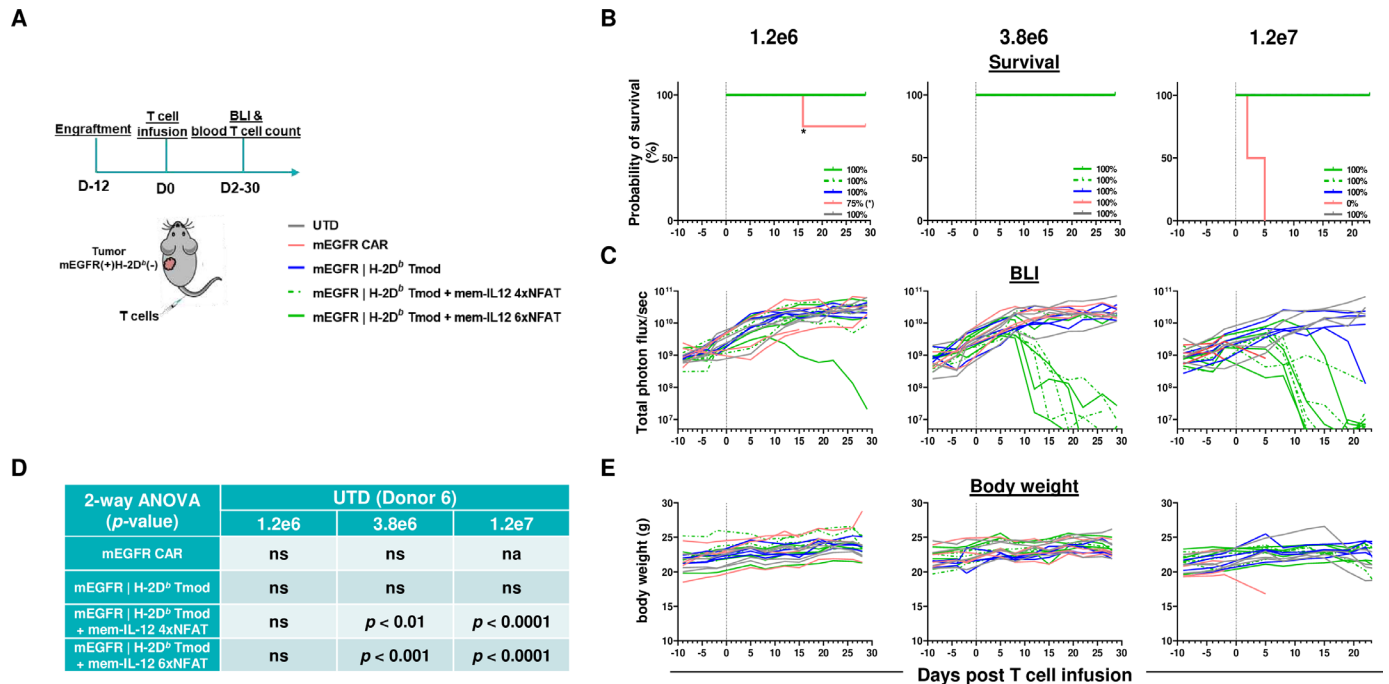


Figure 6 mem-IL-12 boosts Tmod in a surrogate mouse model without evidence of off-tumor toxicity. (A) Schematic of in vivo surrogate animal study. Tumor was established by subcutaneous engraftment of 5×10^4 mEGFR(+)H-2D^b(-) MS751 cells (tumor; left flank); $n=4$ mice per cohort. Tmod potency and selectivity were assessed by (B) Kaplan-Meier curve of survival and percentage of live animals of each group by the end of the study (*perished at blood draw), (C) bioluminescence imaging (BLI) of xenografts, and (E) body weight of mice infused with indicated T cells at indicated effective doses. (D) P values for BLI changes in (C) were calculated using two-way ANOVA. ns: not significant. na: not available. An HLA-A*02(+) donor (Donor 6) was tested. ANOVA, analysis of variance; IL, interleukin; NFAT, nuclear factor of activated T cell; UTD, untransduced.

cells, representing tumor target cells that have undergone LOH.²⁰ mEGFR | H-2D^b Tmod displayed surface expression and in vitro potency and selectivity similar to MSLN | HLA-A*02 Tmod against MS751 (figure 6A; online supplemental figure 6A,B). After infusion into mice, the mEGFR CAR-T alone was highly toxic at the high dose (1.2×10^7), while the mEGFR | H-2D^b Tmod construct was not (figure 6B). Importantly, however, Tmod+inducible mem-IL-12 was active at a $10 \times$ lower dose compared with unboosted Tmod (figure 6C,D). Furthermore, there was no evidence of safety risk based on body weight (figure 6E) and clinical observations (data not shown). All cohorts of mice treated with Tmod+inducible mem-IL-12 survived for the duration of the study and displayed no signs of toxicity. Analysis of mouse blood showed a modest boost by mem-IL-12 in both Tmod cell counts and IFN- γ levels associated with tumor clearance (online supplemental figure S6C,D).

Together with the previous in vivo experiment using dual-flank micro-xenografts and MSLN Tmod, these results suggest that mem-IL-12 boosts the potency of Tmod cells $\sim 10 \times$ with respect to dose-response, without compromising the selectivity window or safety.

mem-IL-12 causes minimal soluble IL-12 release

Though mice treated with Tmod+mem-IL-12 displayed no overt sign of toxicity, we nevertheless analyzed the potential risk of shedding of mem-IL-12 (measured as soluble IL-12p70 heterodimer), both in vitro and in vivo.

No soluble IL-12p70 above baseline level was detected in vitro from either NFAT-driven construct. Although detectable, the EF1 α -driven construct produced soluble IL-12p70 at levels that were 100-fold less than the amount measured from the secreted-IL-12 construct (figure 7A; see online supplemental methods).

Blood from both dual-flank micrograft and surrogate studies described above was also analyzed to measure levels of soluble IL-12p70 in the serum. The EF1 α construct produced persistent detectable levels of IL-12p70 in one mouse 2 weeks after T-cell infusion, suggesting that some mem-IL-12 was cleaved and secreted into the blood; however, IL-12p70 was close to undetectable at any time point in mice treated with NFAT-controlled mem-IL-12 (figure 7B,C). Because the modified IL-12 protein encoded by each construct is identical, this difference likely reflects the higher amount expressed on the surface from the stronger promoter.

DISCUSSION

Because of their key role in T and natural killer cell function, cytokines have been tested as stimulants of potency and persistence for adoptive transfer in the clinic, but with mixed success (see for review²¹). A combination of systemic toxicity and lack of benefit has limited the clinical application of exogenously administered soluble cytokines. For example, exogenous IL-2 has been part of many clinical regimens for solid tumor cell therapy, but

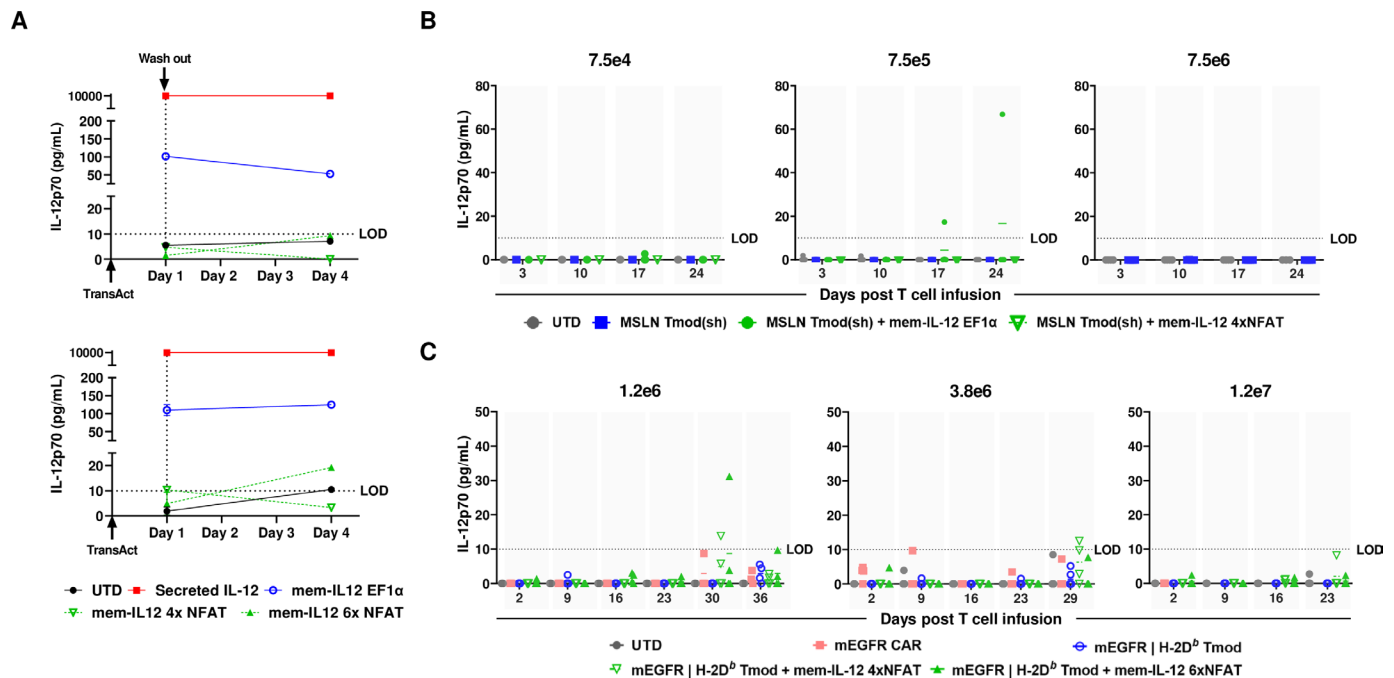


Figure 7 Inducible NFAT-regulated mem-IL-12 produces minimal soluble IL-12 both in vitro and in vivo. (A) T cells were transduced with NFAT-inducible mem-IL-12 lentiviral constructs and activated by TransAct. IL-12p70 level in the supernatant was measured using CBA on day 1 and 4 post-activation. Constitutively expressed mem-IL-12 or secreted IL-12 served as controls. Top=TransAct was washed out on day 1. Bottom=TransAct was left in the culture for 4 days. (B) and (C) Serum IL-12 levels from two in vivo studies (see figures 4 and 5) were measured using CBA. Effective T cell doses (ie, transduced) are listed above the graphs. CAR, chimeric antigen receptor; CBA, Cytometric Bead Array; IL, interleukin; LOD, limit of detection (10 ng/mL); MSLN, mesothelin; NFAT, nuclear factor of activated T cell; UTD, untransduced.

despite a clear effect on the expansion of the infused cells in some cases, its risk versus benefit relationship has been controversial.²² In part to overcome these issues, membrane-tethering has been explored both preclinically and in the clinic. Efforts include engineered expression of membrane-tethered forms of IL-12,^{15 17 23} IL-15,^{24–28} IL-21^{29 30} and both IL-15 and IL-21.^{31 32} Constitutively active common-gamma-chain receptors have also been developed as potential adjuncts to cell therapy.^{19 33 34}

The concept of arming CAR-Ts with IL-12 specifically was introduced well over a decade ago, demonstrating a preclinical potency benefit that involved, among other mechanisms, resistance to inhibition by regulatory T cells.^{35 36} However, clinical trials have shown that CAR-Ts engineered to secrete IL-12, even if regulated by an NFAT promoter, produce toxic levels of IL-12.³⁷ Efforts to refine and better control IL-12-armed CAR-Ts for poorly treated tumor types continue in clinical trials³⁸ (NCT06343376).

In this study, six exogenous cytokines were tested in vitro in Tmod acute cytotoxicity and selectivity assays. Though their effects on acute killing were generally dose-dependent, none overrode selectivity. IL-12, when expressed endogenously in a membrane-tethered form, had significant effects on Tmod efficacy. Moreover, mem-IL-12 preserved the selectivity window of Tmod, a key requirement for a Tmod booster. These results suggest that the other cytokines, despite some mechanistic differences, may offer similar benefits for long-term

Tmod behavior. Nonetheless, at least some of the active constructs tested as membrane-bound signal 3 boosters did not display the same level of benefit as mem-IL-12.

mem-IL-12 provided significant boosts to Tmod long-term potency and persistence in multiple in vitro and in vivo studies. In particular, its quantitative benefits in two substantially different in vivo studies, dual-flank microxenograft and surrogate Tmod models, support the expectation that the mem-IL-12 booster will provide a substantial boost in dose-response of Tmod in humans, possibly up to the 10× increased potency observed in the mouse models. Moreover, the use of NSG mice with human cells may underestimate the actual potency benefit because the injected human T cells do not receive systemic and stromal proinflammatory stimuli to the same extent that they would in humans. Although the inducible NFAT-driven and constitutive EF1α-driven constructs generally showed similar benefits to efficacy and proliferation, the NFAT construct produced lower levels of soluble IL-12 heterodimer, a possible advantage for clinical safety.

Tmod's inherent selectivity is comparable to a benchmark TCR-T (T cell receptor-engineered T cell).³⁹ Nonetheless, numerous factors can interfere with the blocker's effectiveness. These include antigen and receptor levels and the strength of the activation stimulus.⁴⁰ In general, though, the Tmod system is well-buffered against differences in antigen level⁴⁰ and as this work shows, able to handle stimulation by cytokines. To further guard against excessive stimulation by mem-IL-12,

we used a transcriptional control circuit that activates only in response to antigen through an NFAT-regulated promoter.

Even the constitutively expressed signal 3 boosters tested in these studies all displayed antigen dependence, representing another safety benefit. However, it should be noted that this safety advantage may also be a limitation with respect to solid-tumor therapies, where antigen may not be easily accessible in the blood. This limitation may require the engineered cells to access the tumor antigens outside the blood vessel walls before they can respond to signal 3 stimuli. Thus, depending on the characteristics of the manufacturing process and conditioning regimen, activation by antigen may be necessary to bring signal 3 boosters into effect.

METHODS

Cell lines

Cell lines including NCI-H508, MS751, and HeLa were engineered and maintained as previously described.^{12 20 39} Antigen quantification on cell surfaces was done using QIFIKIT (Agilent) following the manufacturer's protocol. Anti-HLA-A*02 (clone BB7.2), anti-CEA (R&D Systems, clone MAB41281), anti-MSLN (R&D Systems, clone MAB32653), anti-mEGFR (R&D Systems, polyclonal), and anti-H-2D^b (Thermo Fisher Scientific, clone B22-249.R1) antibodies were used.

Molecular cloning

Activating and blocking CAR constructs were designed and constructed as previously described.^{12 20 39} Single-chain IL-12 was designed by linking p40 and p35 with a flexible linker (GSGSSRGSGSGSGGGGSK). To form a membrane-tethered IL-12, human B7-1 transmembrane domain was cloned downstream of single-chain IL-12.^{15 18} The mem-IL-12 construct was either cloned into a pLenti vector under an EF1 α promoter, or linked to a synthetic YB-TATA promoter⁴¹ regulated by four or six copies of NFAT response elements (5'-GGAGGAAAACTGTTTCATACAGAAGGCGT-3')¹⁶ and cloned into a pLenti vector in a reverse direction. Constructs for mem-IL-18 and di-IL7R α were designed similarly, with mem-IL-18 fused to B7 TM by a (G4Q)10 linker and ECD and TM domain of IL-7R α replaced with FLAG-tagged CD33 and a constitutively dimerizing form of IL-7R α TM domain termed C7R.¹⁹

Primary T-cell generation, characterization, and functional assessment

Peripheral blood mononuclear cells were purified from Leukopaks purchased from AllCells or HemaCare. Seven different donors were tested, including three HLA-A*02(-) donors (Donor 1–3) and four HLA-A*02(+) donors (Donor 4–7). Detailed methods of CAR-T, Tmod, Tmod+mem-IL-12 generation, characterization, and functional assessment (including activation, acute cytotoxicity assay, tumor

and “normal” mixed co-culture, and long-term antigen-dependent expansion assay) can be found in online supplemental methods.

mem-IL-12 induction and soluble IL-12p70 measurement

0.2e6 T cells, UTD or transduced with various IL-12 constructs were suspended in 200 μ L XVIVO15 media supplemented with 300 IU/mL IL-2 and with or without 1:100 TransAct, then plated in a 96-well flat plate. 24 hours post-activation, one replicate was spun down and washed with XVIVO15 media to remove TransAct, then resuspended in XVIVO15 media supplemented with 300 IU/mL IL-2 and plated in a 96-well flat-bottom plate. On days 1, 2, 3 and 4 post-activation, mem-IL-12 positive T cells were determined by flow cytometry. On days 1 and 4, soluble IL-12p70 concentration in supernatant was analyzed using Cytometric Bead Array (CBA) human IL-12p70 flex set (BD Biosciences), following the manufacturer's protocol.

In vivo mouse studies

In vivo experiments were conducted by Charles Rivers Laboratories under Institutional Animal Care and Use Committee-approved protocols (IACUC protocol number: 2022–1502). For all in vivo studies, female NSG mice (Jackson Labs) 5–6 weeks old were acclimated to the housing environment prior to the initiation of the studies. Details related to the group allocation were not disclosed to Charles Rivers Laboratories.

Animals were implanted subcutaneously with 5e4 MSLN(+)HLA-A*02(-)ffLuc(+) MS751 tumor cells on the left flank and 5e4 MSLN(+)HLA-A*02(+)ffLuc(+) MS751 “normal” cells on the right flank for the dual-flank micro-xenograft model, or 5e4 hEGFR(-) mEGFR(+)H-2D^b(-)ffLuc(+) tumor cells on the left flank for the surrogate Tmod model.²⁰ After 11–12 days, animals were randomized into groups based on BLI of the grafts and rehoused into one cage per treatment group. T cells were administered via the tail vein (intravenous). No animals were excluded after T cell injection. Body weight, clinical parameters, and BLI of the grafts were monitored two times a week for the duration of animal studies. Blood and serum were collected every 7 days starting at 2–3 days post T-cell infusion.

Mouse blood was stained post RBC lysis with anti-mCD45 (clone 30-F11), anti-hCD3 (clone SK7), anti-hCD4 (clone OKT4), and anti-hCD8a (clone RPA-T8) antibodies. Blocker and CAR were stained as described above. Human cytokines in serum samples were measured using CBA human IFN- γ and IL-12p70 flex sets (BD Biosciences).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. All in vitro data are representative of a

minimum of $n=2$ experimental repeats with 2~4 technical replicates per sample, unless otherwise noted. Where applicable, technical replicates are shown as individual data points, with bars denoting means. E:T versus killing curves were fit by a 4-parameter non-linear regression analysis. ET50 values (E:T at 50% specific killing) were interpolated from the curves. Selectivity windows were calculated as ET50 on “normal” target cells divided by ET50 on tumor target cells. Two-way analysis of variance was used to analyze time-dependent differences (ie, BLI in animal studies and fold expansion in repeated tumor exposure), while t-tests were used to analyze differences at individual time points.

Acknowledgements We thank Julyun Oh, Charles Kirsh, Diane Manry, and Agnes Hamburger for helpful suggestions around in vivo study design, Mark Sandberg for troubleshooting flow cytometry, John Welch for helpful discussion, and Chuck Li for technical support with automation and imaging.

Contributors AK is responsible for the overall content as guarantor. AK and JAZ directed the research. JAZ, SI, DL, AZ, SS, JL, RB, JT, AP, MED, and TPR designed and conducted experiments.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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