Armoring NKG2D-based CAR T cells with IL-18 improves in vitro and in vivo anti-tumor activity

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BACKGROUND

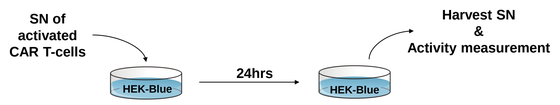
- Whilst delivering impressive clinical efficacy in certain hematological malignancies, Chimeric Antigen Receptor (CAR) T cell therapy has yet to deliver significant clinical impact across a broader array of cancer indications. Armoring CAR T through the co-expression of immune modifying cytokines is an approach that may aid anti-cancer activity and modulation of the immunosuppressive tumor microenvironment (TME) but is currently at an embryonic stage of development. In this study, the potential benefit of expressing IL-18 alongside a NKG2D CAR was assessed.
- NKG2D is an activating receptor mostly expressed by Natural Killer cells and activated T cells. The NKG2D receptor binds 8 different ligands (MICA, MICB, ULBP1-6) that are induced by cellular stress, infections or malignant transformations but rarely detectable on the surface of healthy cells. NKG2D ligands are expressed at the cell surface of malignant cells of various tissue origins, and in syngeneic mouse models they are also expressed in the TME, making them attractive targets for CAR T therapy of both liquid and solid tumors.
- The combination of IL-18 and of our NKG2D-based CAR was evaluated in contexts allowing either autologous or allogeneic CAR-T cell therapy (Fig. 1)

METHODS

- We generated CAR T-cells engineered to express combinations of the following elements:
  - A chimeric receptor consisting of the endogenous NKG2D fused to the cytoplasmic domain of CD3ζ
  - The mature, secreted form of IL-18
  - A cell surface selection marker (truncated CD19, tCD19)
  - A shRNA targeting the NKG2D ligands MICA/MICB to reduce the expression of these CAR targets on the engineered T cells
  - A shRNA targeting CD3ζ (gene name *CD247*) to knockdown cell surface T-cell receptor (TCR) to reduce the risk of graft-versus-host disease (GvHD), which is required for allogeneic CAR-T approaches.
  - A TCR Inhibitory Molecule (TIM)8 peptide, which acts as a dominant negative mutant form of endogenous CD3ζ, that interferes with TCR signaling as an alternative approach to generate allogeneic CAR T cell phenotypes.

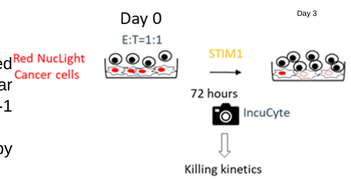
- The required elements were cloned into single retroviral vectors with the expression of the multiple protein transgenic elements achieved by means of 2A proteolytic cleavage sequences.

- ***In vitro* cytokine secretion assays**
  - NKG2D-based CAR T-cells were cultured for 24 hrs alone or with target cells expressing NKG2D ligands (K562 cells) in a 1:1 ratio.
  - Supernatants (SN) from these cultures were harvested for cytokine analysis by ELISA (IFN-γ, IL-18) and *in vitro* IL-18 bioactivity assay.
  - The IL-18 bioactivity assay relies on the HEK-Blue cell line (Invivogen), an IL-18-specific reporter cell line engineered to express an NF-κB/AP-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. Levels of SEAP in SN were measured using the QUANTI-Blue colorimetric detection system (Invivogen).



- ***In vitro* target cell killing assay**

  Single stimulation assay
  - NKG2D-based CAR T-cells were cocultured with target cells expressing a red nuclear fluorescent protein (Red NucLight PANC-1 cells) in a 1:1 ratio for at least 72hrs.
  - Cancer cell killing was monitored by videomicroscopy every 2hrs.



  Restimulation assay
  - NKG2D-based CAR T-cells were cocultured with Red NucLight PANC-1 cells in a 1:1 ratio for 3 rounds of stimulation of 48hrs, 48hrs and 72hrs, respectively.
  - After each stimulation, CAR T-cells are harvested and a new coculture was started with fresh PANC-1 cells.
  - Cancer cell killing was monitored by videomicroscopy every 2hrs.

Fig 1. Secretion of bioactive IL-18 by armored NKG2D-based CAR T-cells

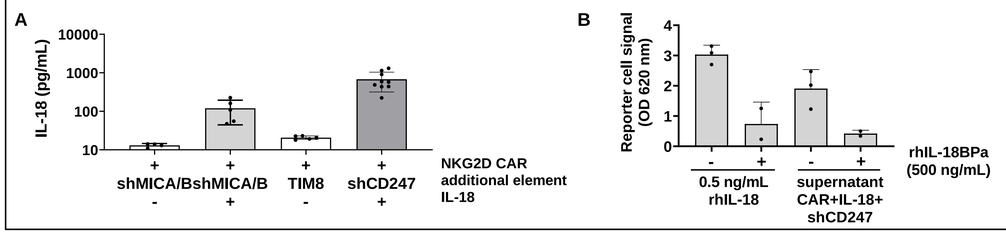


Fig 2. Increased secretion of IFN-γ by IL-18-secreting NKG2D-based CAR T-cells

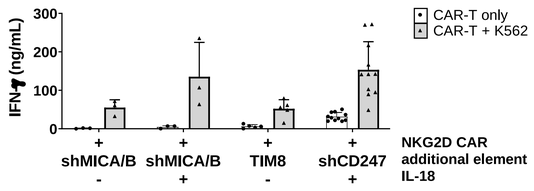
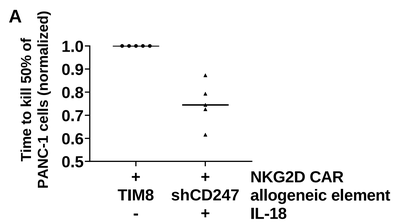


Fig 3. Improved target cell killing by IL-18-secreting NKG2D-based CAR T-cells



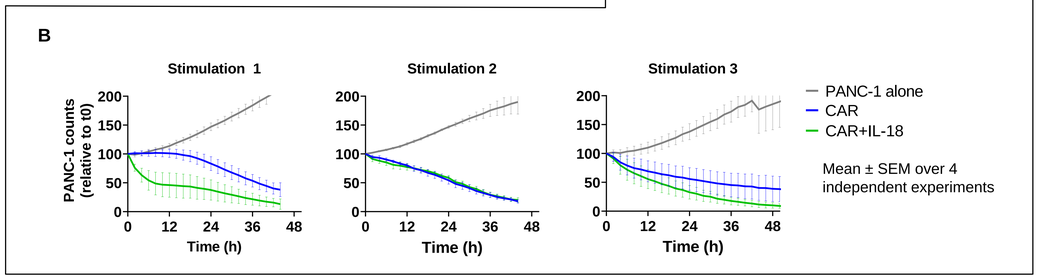
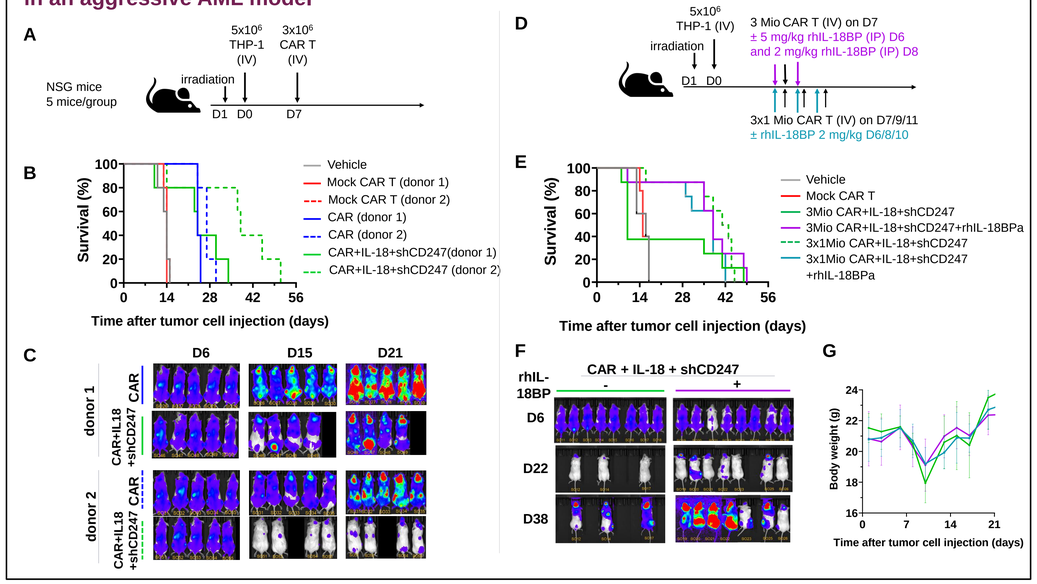


Fig 4. Improved anti-tumor efficacy of non-alloreactive IL-18-secreting NKG2D-based CAR T-cells in an aggressive AML model



RESULTS

Secretion of functional IL-18 by IL-18 armored NKG2D-based CAR T-cells

- During a 24 hour culture period, significantly higher levels of IL-18 were detected in the supernatant of NKG2D-CAR T-cells armored with IL-18 as compared to that of T cells transduced with vectors lacking the IL-18 transgene (Fig. 1A).
- IL-18 produced from IL-18 armored allogeneic NKG2D-based CAR T-cells elicited a response comparable to recombinant IL-18 from the HEK-Blue IL-18-specific reporter cell line. This response was inhibited in presence of recombinant human IL-18 binding protein a (rhIL-18BPa), confirming the bioactivity of the CAR-T derived IL-18 protein (Fig.1B).

IL-18 armored NKG2D-based CAR T-cells display enhanced IFN-γ production upon coculture with target cells

- After incubation with target tumor cells (K562), armored NKG2D-based CAR T-cells produced higher levels of IFN-γ as compared to their non-IL-18 secreting counterparts (Fig. 2).

IL-18 armored NKG2D-based CAR T-cells display enhanced cytotoxicity against target cells *in vitro*

- The cytotoxic activity of NKG2D-based CAR-T cells against PANC-1 cells was followed by videomicroscopy. On average, allogeneic IL-18-secreting NKG2D CAR T-cells killed PANC-1 cells faster than their non-IL18-secreting counterparts (Fig.3A; 27.1±6.4 vs 36.7±11.3 hours).
- The assay was repeated in an iterative design where autologous CAR T were harvested and re-exposed to a fresh culture of PANC-1 cells a further two times. While both NKG2D CAR T cels showed high level; of repeat killing, there was a trend of more rapid killing by the IL-18 armored CAR T-cells (Fig. 3B) and evidence of greater proliferation (data not shown).

IL-18-armored NKG2D-based CAR T-cells display enhanced anti-tumor activity *in vivo* in a xenograft model of acute myeloid leukemia (AML)

- NKG2D-based CAR T-cells generated from 2 donors were administered in an aggressive preclinical model of AML consisting of NOD SCID Gamma (NSG) immunodeficient mice xenografted with THP-1 cells. For each of the 2 donors, the NKG2D-based CAR + IL-18 group showed reduced tumor load by bioluminescence and prolonged survival as compared to Mock or non-IL-18 armored CAR T-cell groups (Fig. 4A-C).

Interplay between IL-18 and IL-18BP

- In certain experiments, a transient decrease in body weight was observed in animals receiving IL18-armored CAR T-cells. This effect was variable from donor to donor (Fig. 4B, E, G).
- IL-18 Binding Protein (IL-18BP) is present at high levels in the peripheral blood of humans, but this protein is clearly absent in mice. Importantly, when recombinant hIL-18BP was administered during the period of CAR-T engraftment, body weight was controlled equivalently to that of the control mice without impacting on the final survival of remaining mice (Fig. 4D-G).

CONCLUSION AND DISCUSSION

- Armoring autologous and allogeneic NKG2D-based CAR T-cells with IL-18 resulted in:
  - *in vitro* secretion of bioactive IL-18;
  - increased *in vitro* secretion of IFN-γ upon coculture with target cancer cells;
  - Increased and prolonged *in vitro* cytotoxic activity against target cancer cells;
  - prolonged survival and diminished tumor load in an aggressive *in vivo* AML model.
- In THP-1-xenografted NSG mice, the transient bodyweight reduction sometimes observed after injection of IL-18 secreting CAR-T cells was alleviated when mice were administered rhIL-18BPa. Of note, multiple clinical trials have demonstrated that in cancer patients, repeated administration of high doses of rhIL-18 is safe and drives an increase of circulating IL-18BP [1-3].
- Together, these observations imply that armoring NKG2D CAR T-cells with IL-18 is likely to drive improved anti-tumor activity of the CAR T cells, in line with previous publications [4,5], while the presence of systemic IL-18BP [6] should negate possible toxicities arising from high level constitutive expression of the cytokine.

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