Discovery Of Lineage Specific Regulatory Elements For Development Of *In Vivo* CAR Immune Cell Therapy Via HSC Engineering

Alvin Pratama, Dhruv Varshney, Dennis Ramos Trinidad, Ying-Cing Lin, Cristina Santoriello, Chirayu Chokshi, Yiwen Zhao, Richard Davidson, Charles Fox, Victoria Blake, Sophia Chan, Adam Golding, Alexey Seregin, Chapman Wright, Joe Salas, Robert Peters, Corinne Decker, and Yinghua Wang

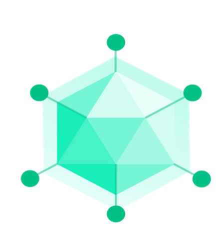
Ensoma, Inc., Boston MA, USA

Abstract

Background Chimeric antigen receptor T (CAR-T) cell therapy has shown limited efficacy in solid tumors, largely due to the immunosuppressive tumor microenvironment (TME) and inefficient trafficking of CAR-T cells to tumor sites. CAR-engineered macrophages (CAR-M) can remodel the TME and promote lymphocyte infiltration but have yet to demonstrate meaningful clinical benefit. Engineering multiple cell types to fully harness the synergy between innate and adaptive immunity poses a promising strategy for solid tumor treatment. **Methods** We developed a virus-like particles (VLPs) platform based on helper-dependent adenovirus to enable *in vivo* engineering of hematopoietic stem and progenitor cells (HSPCs) via targeting of CD46. These VLPs have a cargo capacity of up to 35 kilobases, enabling delivery of multi-cellular CAR under distinct lineage-specific promoters for precise immune cell engineering. **Results** To achieve selective therapeutic payload expression, we systematically screened and identified promoters that drive robust, lineage-restricted CAR expression in effector immune cells. The promoters were ranked based on their strength in T and NK cells with minimal expression in B cells and HSPCs. We also identified a promoter that confined CAR expression to mature myeloid lineage. CAR constructs under these promoters were validated *in vitro* using primary human and murine cells, generating functional CAR-T, CAR-NK, and CAR-M cells with potent, antigen-dependent cytotoxicity. To assess expression *in vivo*, HSPCs from hCD46 transgenic mice were transduced with VLPs encoding CARs under either ubiquitous (CAG) or lineage-restricted promoters and then transferred into irradiated C57BL/6 mice. While the CAG promoter drove CAR expression across all immune lineages, T/NK- and myeloid-restricted regulatory elements promoted CAR expression selectively in their respective lineages.

Our platform for *in vivo* gene therapy

Virus-like particle (VLP)



Evolved capsid
Adenoviral vector built on evolved, high efficiency gene delivery vectors

"Gutless" vector
Contains no viral genes, resulting in high payload capacity

Hematopoietic Tropism
Highly preferential transduction of HSCs & derived lineages

35 kB Payload Capacity
Enables multiplexed gene insertion controlled by distinct regulatory elements

Multi-lineage CAR strategy for Immuno-Oncology

- **Multiple immune cell types** engineered *in vivo*
- **Regulatory elements** enable cell type-specific multiplexing of anti-tumor modalities
- **Self-renewing** source of HSC-derived engineered effector cells

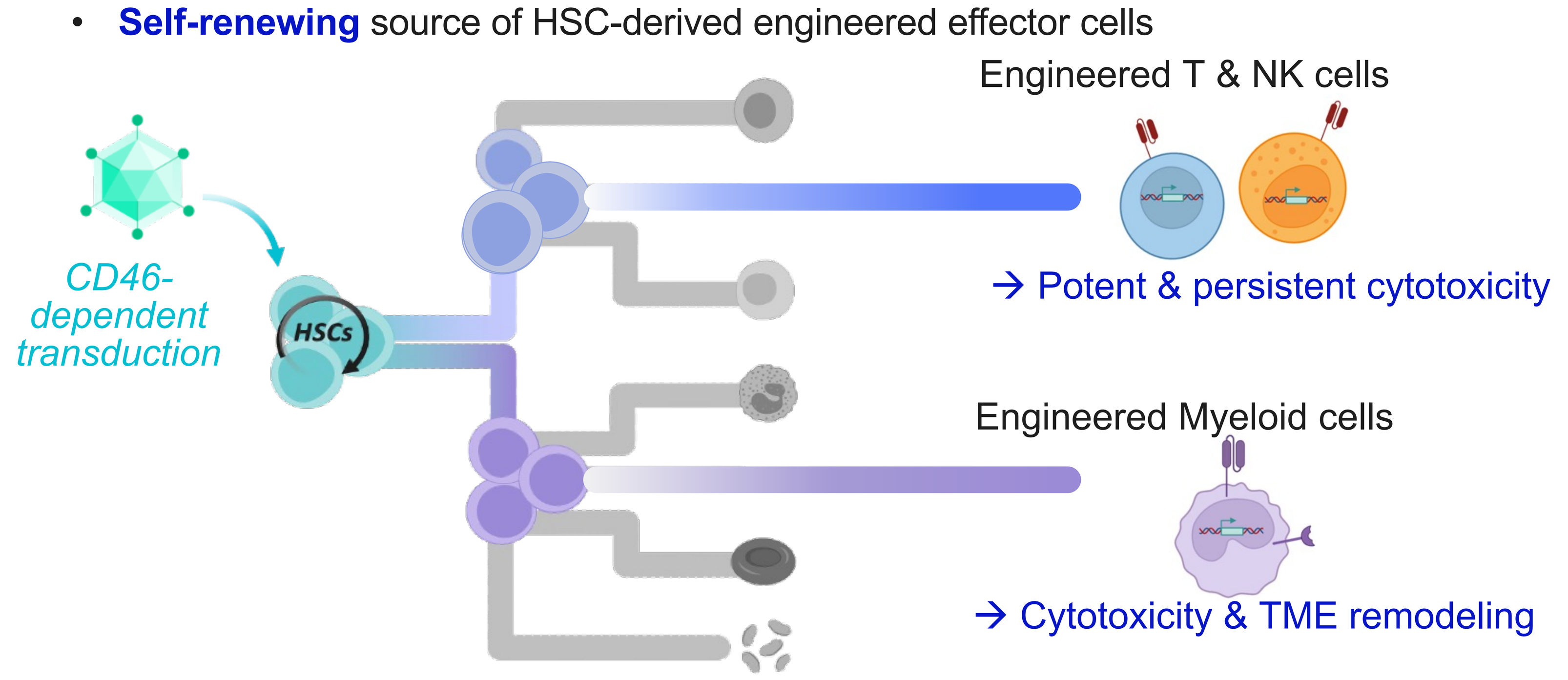


Figure 1. Mobilization of HSCs into peripheral blood enables *in vivo* VLP targeting and transduction. Direct transduction of circulating myeloid, T, and NK cells may generate a population of armed effector cells within days of VLP administration. Transduced HSCs home to the bone marrow where integrated HSCs give rise to engineered immune cell lineages. Long-term HSCs comprise a self-renewing pool of effector cells, conferring potentially durable anti-tumor activity from a single VLP dose.

Screening of T/NK cell-specific promoters for robust CAR expression and effective tumor cell killing *in vitro*

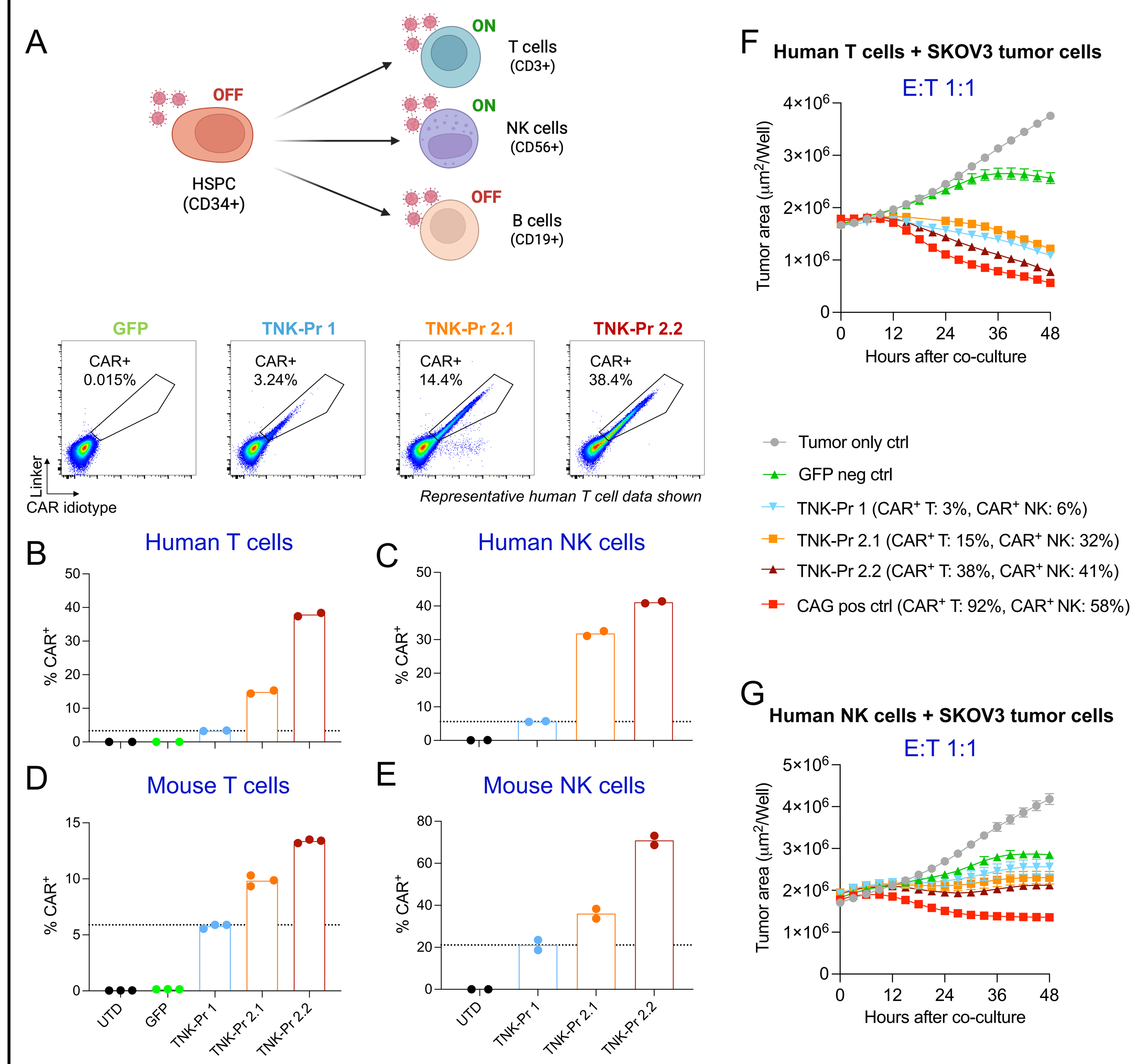


Figure 2. (A) Screening strategy to find strong and specific promoters to drive CAR expression in T and NK cells. **(B-E)** *In vitro* HER2 CAR expression in human T cells (B), human NK cells (C), mouse T cells (D), and mouse NK cells (E) driven by select T/NK cell promoters. Representative flow plots showing HER2 CAR expression on human T cells are shown as examples. Data are representative of 2 donors. Untransduced (UTD) and GFP-transduced controls are shown as negative controls. Bar graphs represent the mean. **(F-G)** HER2⁺ SKOV3 tumor cell line growth curves when co-cultured with either human T cells (F) or human NK cells (G) that were transduced with VLPs encoding HER2 CAR driven by CAG or T/NK-specific promoters (TNK-Pr). E:T ratio was calculated based on total T or NK cell numbers.

T/NK cell-specific promoters show minimal expression in HSPCs and other mature immune cells

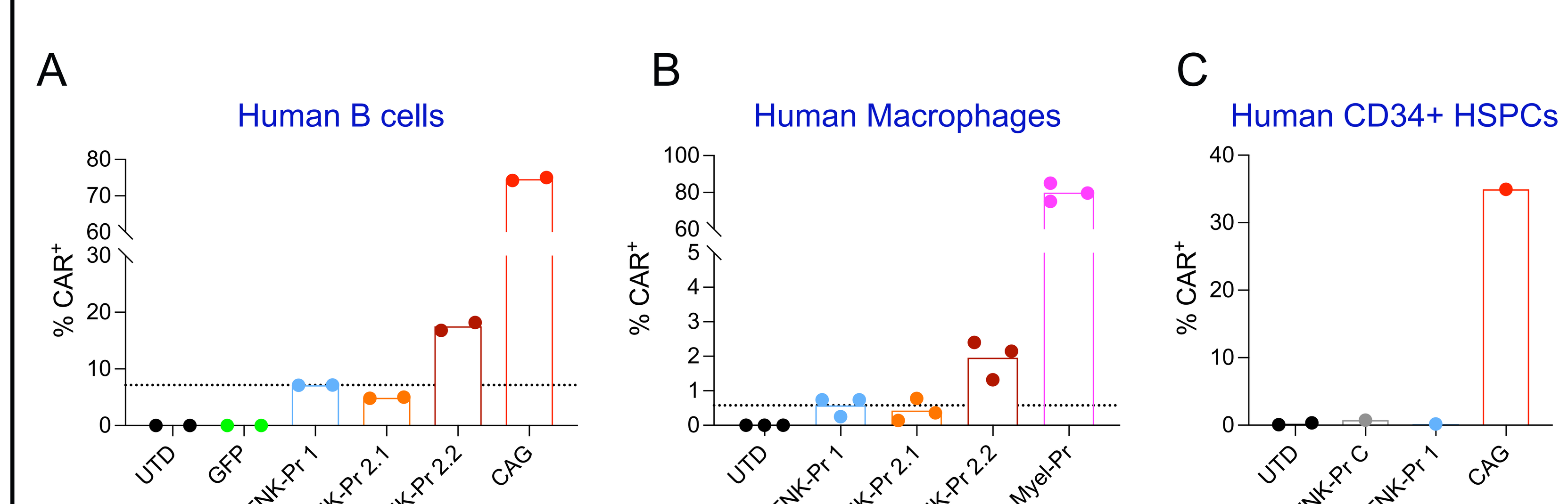


Figure 3. (A-C) *In vitro* HER2 CAR expression in human B cells (A), human macrophages (B), and human CD34+ HSPCs (C) driven by CAG or several T/NK cell promoters (TNK-Pr). Data are representative of 2 donors. Untransduced (UTD) and GFP-transduced samples are shown as negative controls. Bar graphs represent the mean.

Myeloid lineage-specific promoter drives strong and potent CAR expression in human macrophages *in vitro*

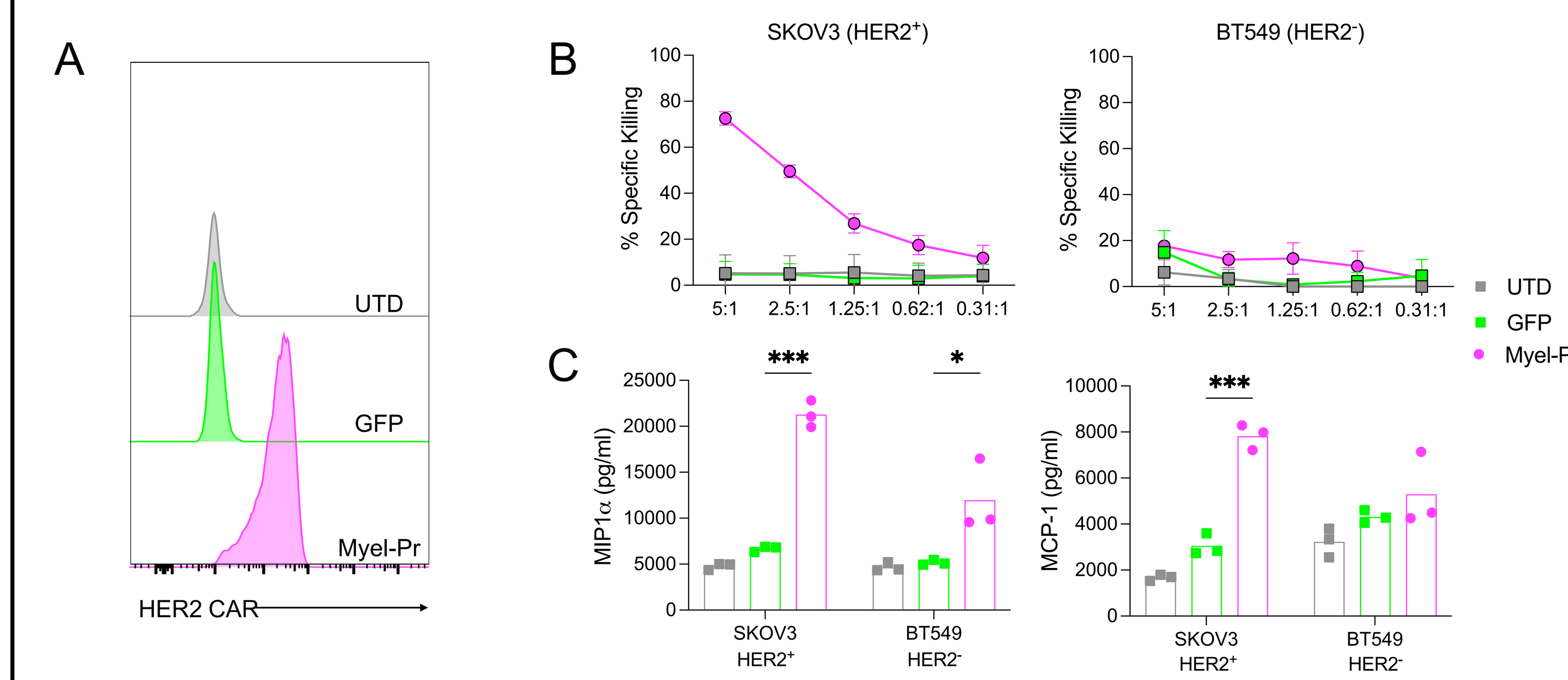


Figure 4. (A) Histogram showing HER2 CAR expression driven by the myeloid-specific promoter (Myel-Pr). Data are representative of 4 donors. **(B)** *In vitro* cytotoxicity of human macrophages expressing HER2 CAR assessed 48 hrs after co-culture with HER2⁺ SKOV3 tumor cells (left) or with HER2⁻ BT549 tumor cells (right) at stated E:T ratios. **(C)** MIP1α (left) and MCP1 (right) chemokine levels in the supernatant of human macrophages and tumor cell line co-cultures as outlined in (B). Supernatants were collected 24 hrs after co-culture and analyzed by MSD. Statistical significance was calculated by unpaired *t* tests.

Lineage-specific promoters are restricting CAR expression to their respective cells *in vivo*

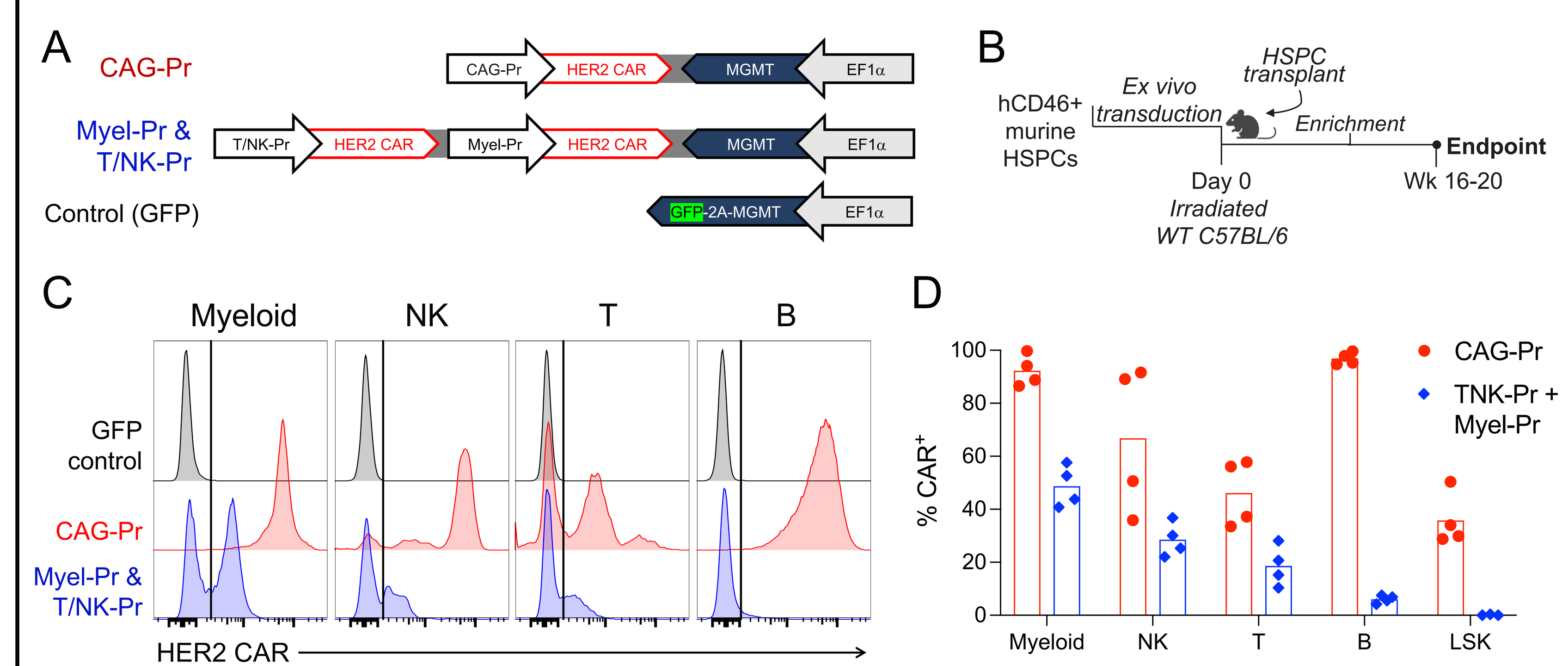


Figure 5. (A) VLP vector encoding anti-HER2 CAR driven by ubiquitous (CAG) or T/NK- and myeloid-specific promoters, along with an EF1α driven $MGMT^{P140K}$ cassette to enable selective enrichment of integrated cells. **(B)** Schematic of *ex vivo* VLP treatment of hCD46+ murine HSPCs and transplant into irradiated C57BL/6 mice. **(C-D)** Fluorescence histogram (C) and frequency (D) of HER2 CAR+ cells in blood (and in bone marrow for LSK cells) at 16 weeks post-HSPC transplant. Bar graphs represent the mean.

Conclusions

- *In vitro* screening identified several **robust and specific T/NK cell promoters** to drive HER2 CAR expression
- Human **macrophages show abundant HER2 CAR expression** driven by myeloid-specific promoter
- Lineage-specific promoters direct CAR expression to discreet mature immune cells, enabling **regulated expression of multiplexed therapeutic payloads**

Related posters

Poster **302**: Yiwen Zhao *et al. In vivo* HSC engineering with VLPs generates lineage-restricted, multiplexed CAR-M, NK, and T cells to cooperatively mediate robust and durable solid tumor control in pre-clinical models