Targeting NRP-1 function for improved CAR T cell therapy

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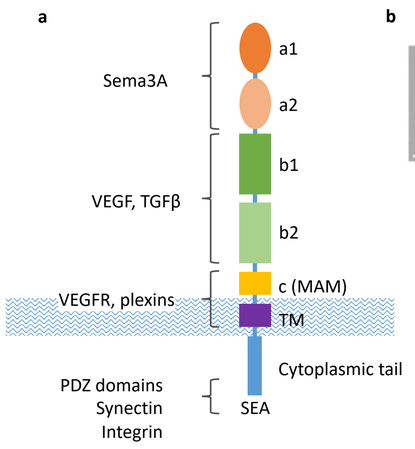
Abstract

Neuropilin-1 (NRP-1) is a transmembrane glycoprotein expressed in several immune cell types, including T cells, where it localizes to the immune synapse and actively inhibits TCR signaling. Previous studies in mice showed that inhibition of NRP1 by blocking antibodies enhanced antitumor efficacy, while genetic ablation of NRP1 synergised with PD-1 blockade to promote tumor rejection.

We hypothesized that knocking out NRP-1 in human Chimeric Antigen Receptor (CAR) T cells, using CRISPR/Cas9 technology, would enhance their function. However, initial results in *in vitro* spheroid assays and in an *in vivo* xenogeneic orthotopic model showed that NRP-1 KO CAR T cells failed to outperform NRP-1 WT CAR cells, and CAR T cell function seemed impeded by NRP-1 knock-out.

We are investigating these contradictory effects of NRP-1 in CAR T cell and regular T cell function by comparing the immune synapse formed between target tumor cells and CAR T cells or regular T cells. In particular, we will study the localization and interactions of NRP-1 at these synapses using confocal microscopy.

We also have hypothesized that the intracellular and extracellular components of NRP-1 may have contradictory roles, in forming or stabilizing the immune synapse and in T cell signaling. We will study the effects of these separate components on CAR T cell function using truncated or hybrid NRP-1molecules. These next steps will allow us to elucidate NRP-1 function in CAR T cells and enable us to design enhanced cell therapy approaches.



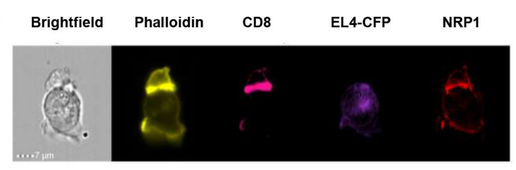


Figure 1. a. Schematic structure of NRP-1 and ligands of each domain. **b.** Localisation of NRP-1 at the immune synapse between activated OT1 CD8 T cells and EL4-CFP-OVA tumor cells, observed by ImageStream (Rossignol *et al.*, iScience 2022).

1. Deletion of NRP-1 compromises CAR T cell function *in vitro* and *in vivo*

A. NRP1-KO CAR T cells were generated by Crispr/Cas9 targeting of the *NRP-1* gene and lentiviral transduction of 19-BBz CAR

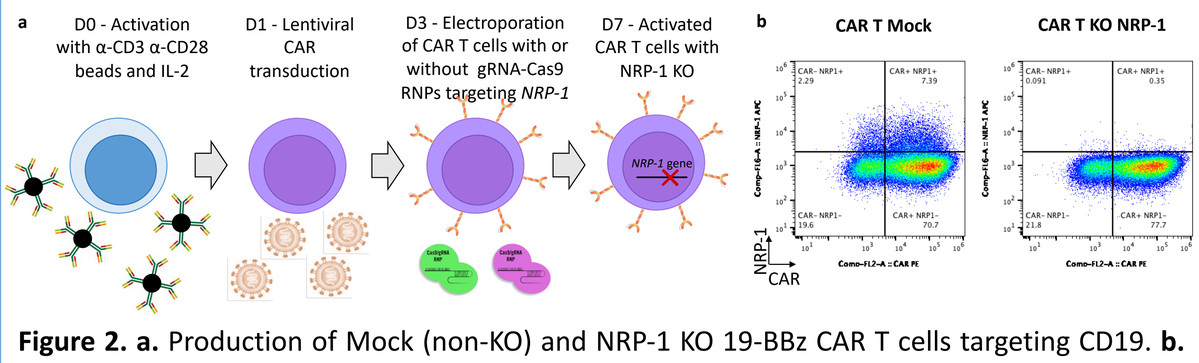


Figure 2. a. Production of Mock (non-KO) and NRP-1 KO 19-BBz CAR T cells targeting CD19. **b.** CAR expression and NRP-1 KO were validated by FACS and/or by sequencing, with restimulations required to increase NRP-1 expression in Mock CAR T cells.

B. *In vitro* spheroid model:
CAR T cells with NRP-1 KO were less effective in eliminating target cell spheroids

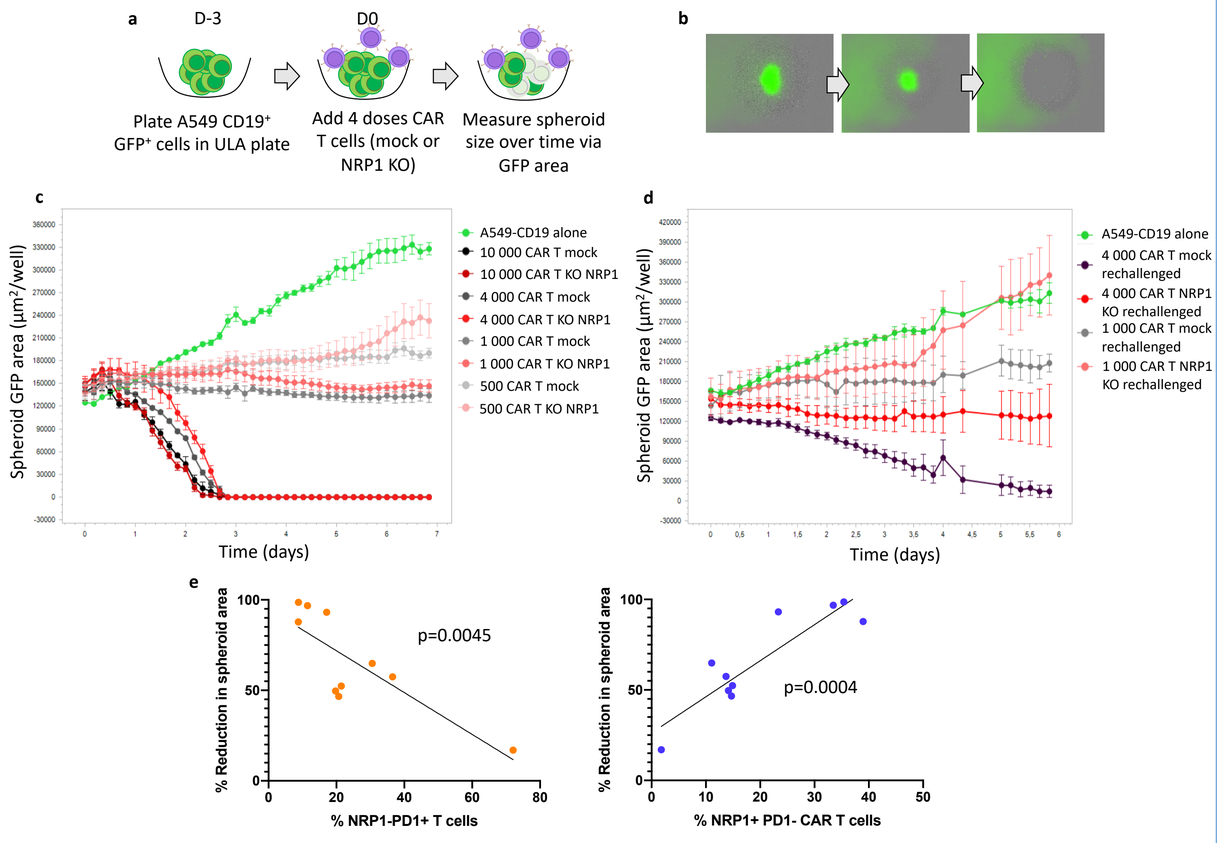


Figure 3. a. Experimental setup for evaluating NRP-1 KO CAR T cell efficacy *in vitro*. **b.** Spheroid (green) shrinks as target cells are killed by effector cells. **c.** Spheroid size measured by fluorescence in co-culture with 4 different doses of Mock or NRP-1 KO CAR T cells. **d.** Spheroid size in a re-challenge co-culture, with CAR T cells that have already eliminated another spheroid. **e.** In an experiment with several different co-culture conditions, CAR T cell cytotoxic activity correlated positively with the proportion of NRP-1^{+}PD-1^{-} cells, and negatively with the proportion of NRP-1^{-}PD-1^{+} cells.

C. *In vivo* NSG mouse model:
Mice that received NRP-1 KO CAR T cells had impaired tumor control and were more prone to relapse

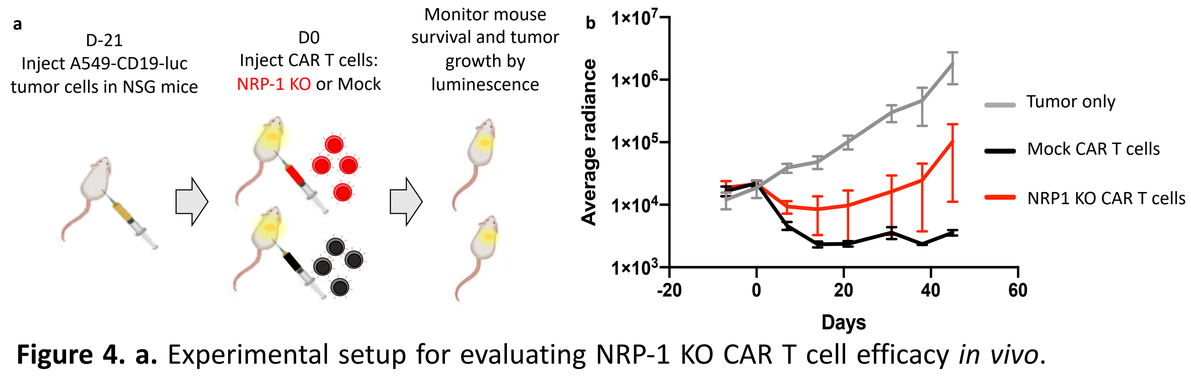


Figure 4. a. Experimental setup for evaluating NRP-1 KO CAR T cell efficacy *in vivo*. **b.** Tumor growth measured by luminescence in mice that received 0.3 million either Mock or NRP-1 KO CAR T cells.

2. What are the roles of NRP-1 in TCR- and CAR-mediated synapses?

- Is NRP-1 required for CAR-mediated immune synapse formation?
- Does NRP1 co-localise with PD-1 in the CAR-mediated synapse?

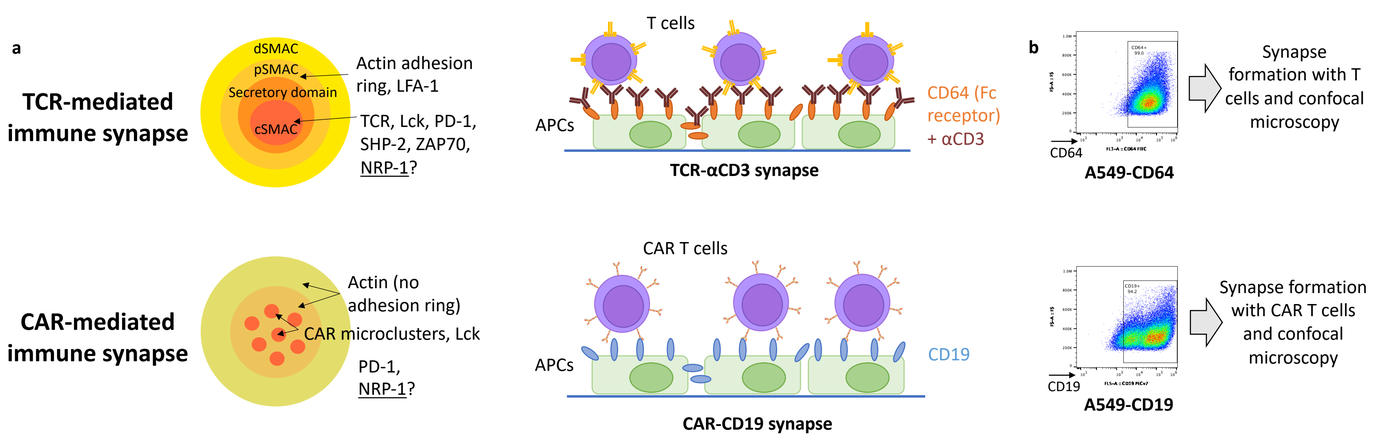


Figure 4. a. Schematic TCR- and CAR-mediated immune synapses. **b.** Generation of antigen-presenting cells for observation of synapses by confocal microscopy.

3. Conclusions & Perspectives

While NRP-1 deletion or inhibition enhances the activity of ordinary T cells, this appears on the contrary to compromise CAR T cell function both in an *in vitro* spheroid model, and an *in vivo* NSG mouse model using A549-CD19 as a tumoral target. Additionally, CAR T cell cytotoxic activity correlated positively with cell populations expressing NRP-1 without PD-1.

We have hypothesized that the extracellular and intracellular domains of NRP-1 could have contradictory roles, depending on context, levels of activation, and other molecules interacting with NRP-1.

We have also hypothesized that NRP-1 could play differing roles in the TCR- and CAR-mediated immune synapses. We are setting up confocal microscopy experiments to compare the localizations and interactions of NRP1 in these different contexts.

Untangling the roles and mechanisms of action of NRP-1 could eventually allow us to target this protein for improved CAR T cell therapy.