CAR-T cell Engineering Strategies Aimed at Safe and Effective Targeting of Solid Tumors

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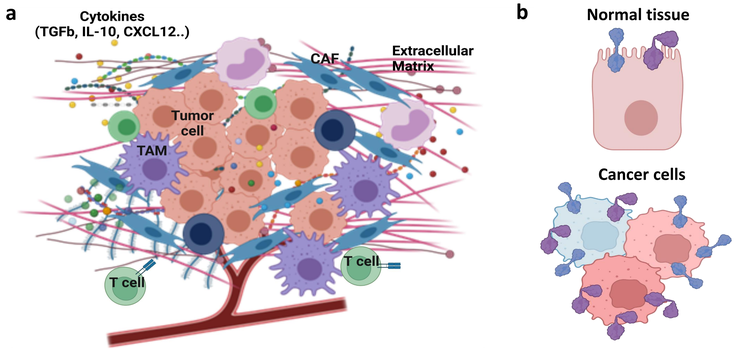
#1 Introduction: The Solid Tumor Microenvironment

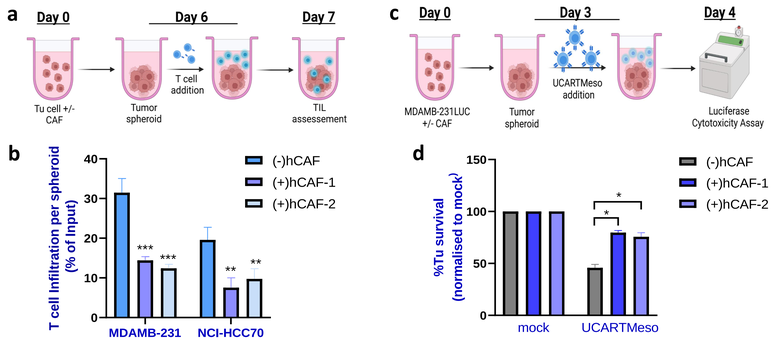
Adoptive cell therapy based on chimeric antigen receptor-engineered T (CAR-T) cells has been transformational for selective heme malignancies. However, its therapeutic efficacy in solid tumors is severely hampered by several factors. Prominent among these is a complex tumor microenvironment (TME), the components of which subvert immune clearance by **inhibiting intra-tumor T cell infiltration** and establishing an **immunosuppressive milieu**. Furthermore, **tumor antigen heterogeneity** as well as low level expression of CAR-directed tumor-associated antigens (TAA) in normal tissues can result in **antigen-escape** and **"on-target off-tumor"** cytotoxicity respectively, raising significant concerns about therapeutic safety and relapse.

(**a**) Pictorial representation of the solid tumor microenvironment. (**b**) Schematic representation of heterogenous antigen expression in normal and cancer cells.



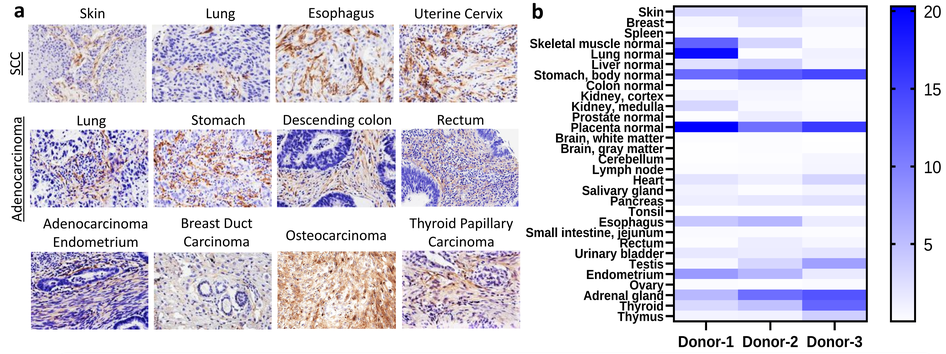
#2 Cancer-associated Fibroblasts inhibit T cell infiltration and CAR-T cytotoxicity against solid tumors

(**a**) Schematic of T cell intra-spheroid infiltration assay. (**b**) Quantitation of T cell infiltration in tumor spheroids alone or mixed with patient TNBC-derived CAFs, as a percentage of input. (**c**) Schematic of MesothelinCAR; $TRAC_{KO}$ T (UCARTMeso) cells cytotoxicity assay against MDAMB-231 spheroids alone or mixed with patient TNBC-derived CAFs. (**d**) Quantitation of UCARTMeso anti-tumor cytotoxicity assay elicited in (**c**).



#3 Fibroblast Activation Protein (FAP) expressed on CAFs is predominantly localized to the TME

(**a**) Immunohistochemical staining of FAP expression in patient tumor samples (**b**) Quantitation of FAP expression on normal human tissue microarray, heat map represents percentage positive area per section (three donors).

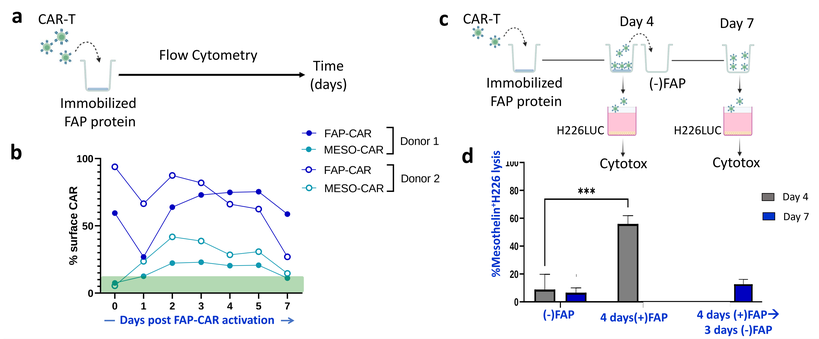


#4 Combating "cold tumors" and "on-target, off-tumor" cytotoxicity with TALEN® edited Dual Inducible CAR-T cells

(**a**) Schematic of engineering dual inducible CAR-T cells targeting FAP[+]Mesothelin[+] tumors. (**b**) Pictorial representation of allogeneic dual inducible rLv-FAPCAR,$TRAC_{KO}PD1_{MesoCAR}$ T cell. (**c**) Phenotype of TALEN® engineered dual inducible rLv-FAPCAR,$TRAC_{KO}PD1_{MesoCAR}$ T cells by flow cytometry. (**d**) Graphical depiction of percentage Mesothelin CAR integration at CAR-inducible PD-1 locus, measured by ddPCR.

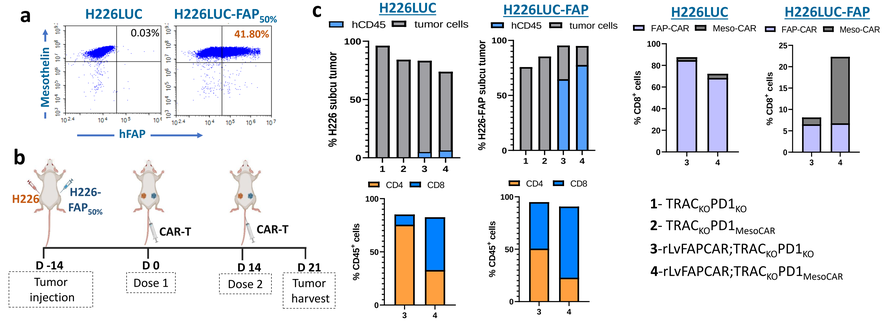
#5 MesoCAR expression and activity is stringently regulated by FAPCAR engagement

(**a**) Schematic of Dual Inducible rLv-FAPCAR;$TRAC_{KO}PD1_{MesoCAR}$ T cell activation with FAP protein. (**b**) Flow cytometry analysis of cells from (**a**) for FAP-CAR and Meso-CAR expression (**c**) Schematic for assessing MesoCAR activity against Mesothelin[+]FAP[-] H226LUC tumor cells upon FAP-CAR activation and subsequent disengagement. (**d**) Quantitation of cytotoxicity of MesoCAR elicited in (**c**).



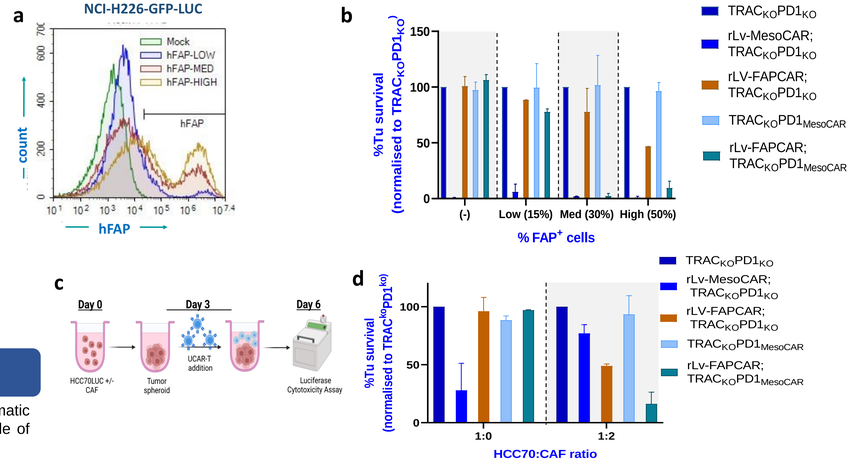
#6 MesoCAR expression and activity is restricted to FAP[+] tumors

(**a**) Flow cytometry plots Mesothelin[+] tumor cell line NCI-H226-LUC transduced to express human FAP protein. (**b**) Schematic of *in vivo* mouse study to assess specificity of FAPCAR-dependent MesoCAR expression and activity. (**c**) Cellular profile of H226 or H226-FAP tumors treated as in (**b**), as determined by flow cytometry.



#7 rLv-FAPCAR;$TRAC_{KO}PD1_{MesoCAR}$ T cells display superior dual CAR killing against FAP[+]Mesothelin[+] tumor spheroids with minimal 'on-target off-tumor' cytotoxicity

(**a**) Mesothelioma cell line NCI-H226-LUC transduced to express human FAP protein at different cellular abundance. (**b**) Graphical representation of CAR-T cytotoxicity against 3-D spheroids formed by tumor cells from (**a**). (**c**) Schematic of CAR-T cytotoxicity assay against 3-D spheroids of TNBC cell line HCC70LUC alone or mixed with TNBC-derived CAFs. (**d**) Graph depicting results of CAR-T cytotoxicity assay outlined in (**c**).



Conclusions

1. **Dual Inducible rLv-FAPCAR;$TRAC_{KO}PD1_{MesoCAR}$ T cells display higher cytotoxicity against FAP[+]Mesothelin[+] tumors than either of the single CAR-T cells alone.**
2. **CAR-targeting of CAFs increases cytotoxicity of tumor cell-targeting CAR in physiologically relevant models of solid tumors.**
3. **Dual inducible rLv-FAPCAR;$TRAC_{KO}PD1_{MesoCAR}$ T cells are unable to kill Mesothelin[+] tumors with lower than physiological FAP[+] cellular abundance, exhibiting limited 'on-target off-tumor' toxicity.**
4. **TALEN® engineered Dual Inducible CAR-T strategy of constitutive TSA-CAR inducing expression of TAA-CAR can increase solid tumor targeting efficacy while limiting off-tumor toxicities.**