

Supplemental Information and Figures

A therapeutic T cell receptor mimic antibody targets tumor-associated PRAME peptide / HLA-I antigens

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Supplemental Materials and Methods

Selection and Characterization of scFv Specific for PRAME Peptide/HLA-A2 Complexes

A human scFv antibody phage display library was used for the selection of mAb clones. In order to reduce the conformational change of HLA-A2 complex introduced by immobilization onto plastic surfaces, a solution panning method was used in place of conventional plate panning. In brief, biotinylated negative antigens RHAMM-R3/HLA-A2 were first mixed with the human scFv phage library (7×10^{10} clones), then the antigen-scFv phage antibody complexes were pulled down by streptavidin-conjugated Dynabeads M-280 through a magnetic rack and discarded. This step removed phage that bound to HLA-A2 along or HLA-A2 in complex with an irrelevant peptide. A repeat of this process on the positive ALY/HLA-A2 monomers was then conducted. ALY/HLA-A2-bound clones were then eluted and were used to infect *E. Coli* XL1-Blue. The scFv phage clones were expressed in the bacteria and were purified (1). Panning was performed for 3-4 cycles to enrich scFv phage clones binding to ALY/HLA-A2 complex specifically. Positive phage clones were determined by standard ELISA method against biotinylated single chain HLA-A2/ALY peptide complexes. A total of 25 positive clones that possessed unique DNA coding sequences were subjected to further characterization. Binding to peptide/HLA-A2 complexes on live cell surfaces was determined using a TAP-deficient, HLA-A2+ cell line, T2. T2 cells were pulsed with peptides (50 $\mu\text{g}/\text{mL}$) in the serum-free RPMI1640 medium, in the presence of 20 $\mu\text{g}/\text{mL}$ β 2M overnight. The cells were washed, and the staining was performed in following steps. The cells were first stained with purified scFv phage clones, and followed by staining with a mouse anti-M13 phage coat protein mAb (Invitrogen MA1-12900), and finally the goat F(ab)₂ anti-mouse IgG conjugated to A488 (Invitrogen A11017) before flow cytometry. Each step of the staining was done between 30-60 minutes on ice and the cells were washed twice between each step of the staining. 4 clones (Pr8, Pr17, Pr20, Pr29) showed robust and specific binding to T2 cells pulsed with ALY peptide but not to T2 cells left unpulsed or pulsed with the irrelevant control RHAMM-3 peptide, and were engineered into full length human mAb.

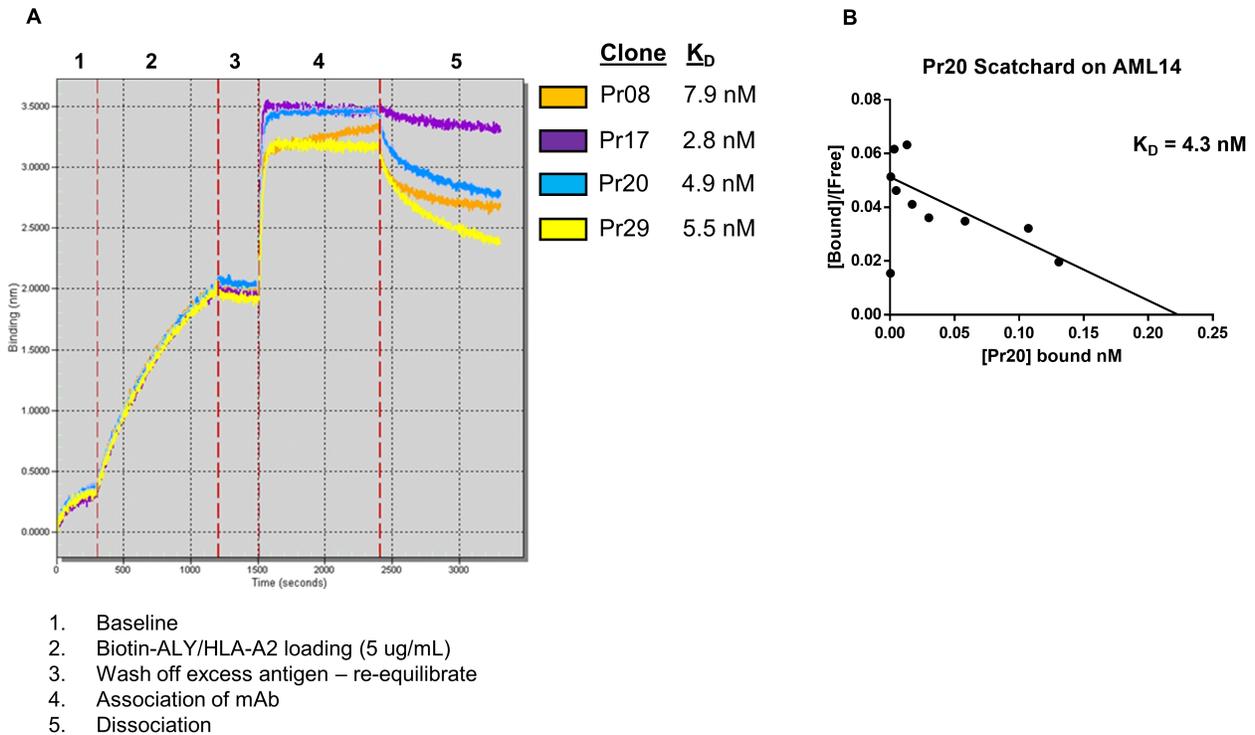
Engineering Full Length mAb Using the Selected scFv Fragments.

Full-length human IgG1 of the selected phage clones were produced in HEK293 and Chinese hamster ovary (CHO) cell lines, as described (2). In brief, antibody variable regions were sub-cloned into mammalian expression vectors, with matching human lambda or kappa light chain constant region and human IgG1 constant region sequences. Afucosylated Pr20M was produced in a similar method except in modified CHO cells as described (3). Molecular weights of the purified full-length IgG antibodies were measured under both reducing and non-reducing conditions by electrophoresis.

Selection of Pr20 as Lead mAb Clone

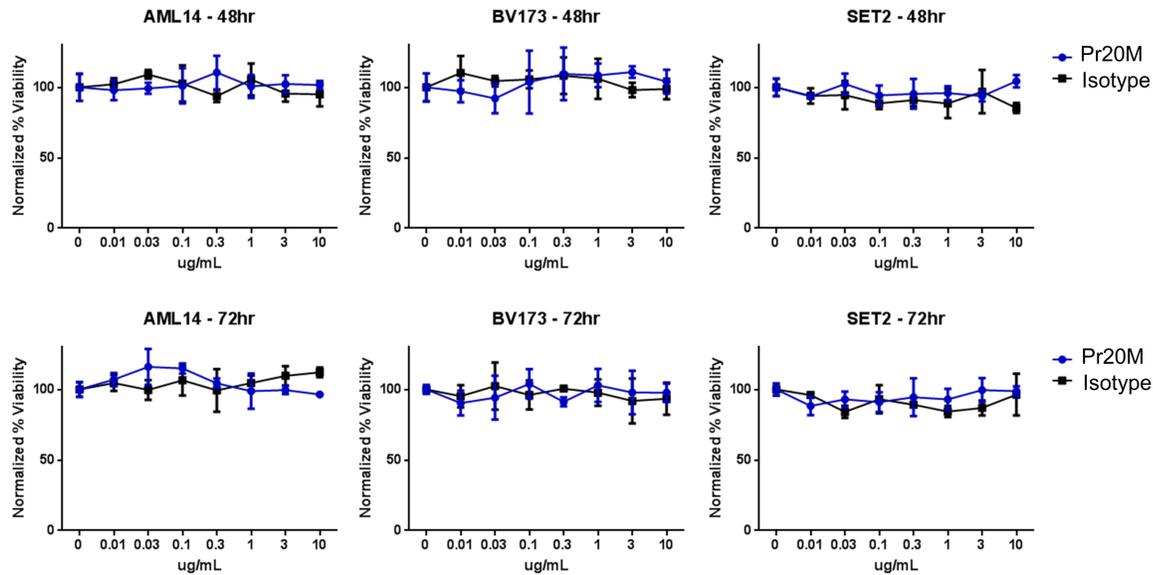
4 full-length human IgG1 clones (Pr8, Pr17, Pr20, Pr29) were assayed for binding to HLA-A2+ healthy donor PBMC populations and PRAME+/HLA-A2+ leukemia cell lines AML14 and BV173. Pr20 was chosen as the lead TCRm mAb clone due to robust binding to cell lines without substantial binding to healthy PBMC populations. Other clones were not pursued due to either non-specific binding to HLA-A2+ healthy donor PBMC populations or inability to bind or mediate ADCC against PRAME+/HLA-A2+ tumor cells, suggesting that these clones did not bind endogenously processed and presented ALY/HLA-A2 complexes which may have subtle structural constraints compared to exogenously-loaded ALY on HLA-A2 in the T2 cell assay or monomers folded *in vitro*.

1. Persic L, Roberts A, Wilton J, Cattaneo A, Bradbury A, Hoogenboom HR. An integrated vector system for the eukaryotic expression of antibodies or their fragments after selection from phage display libraries. *Gene*. 1997;187:9-18.
2. Caron BPC, Laird W, Co MS, Avdalovic NM, Queen C, Scheinberg DA. Engineered Humanized Dimeric Forms of IgG Are More Effective Antibodies. *J Exp Med*. 1992;176:1191-1195.
3. Liu C, Xiang J, Yan S, Wang P, Tai C. Modified host cells and uses thereof. U.S. Patent 2010/0081195. 2010.



Supplemental Figure 1. Pr20 exhibits a low nM affinity for ALY/HLA-A2.

(A) A Fortebio device was used to determine the affinity of Pr20 for the target protein complex ALY/HLA-A2 monomer. After biotinylated ALY/HLA-A2 monomers were loaded (2), excess antigen was washed off and the instrument was re-equilibrated (3). The association phase (4) and dissociation phase (5) data are shown. (B) Scatchard analysis was performed on the PRAME+/HLA-A2+ AML14 cells using increasing concentrations of 125 -Pr20. Affinity of Pr20 to the complex and avidity of Pr20 TCRm to AML14 was confirmed to be in the low nM range by the assays, respectively. Experiment in (A) was performed once and experiment in (B) was performed 2 times with 3 technical replicates per experiment.



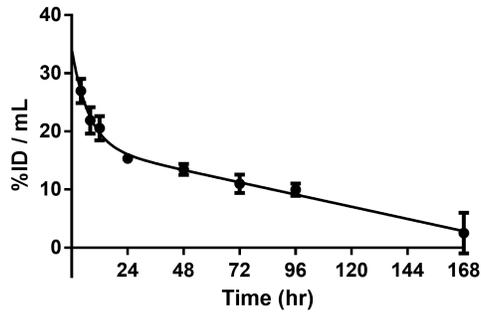
Supplemental Figure 2. Pr20M does not mediate direct cytotoxicity or growth inhibition on PRAME+/HLA-A2+ leukemia.

PRAME+/HLA-A2+ leukemia cells were incubated with the indicated concentration of Pr20M for 48 or 72 hours. An ATP-based viability assay (Promega – CellTiter Glo) was used to determine relative cell viability compared to cells incubated with an isotype control antibody. The assay was performed as recommended by manufacturer. Data is representative of 3 experiments with 3 technical replicates for each dose and time point.

A

WT Mice

Pr20M Pharmacokinetics

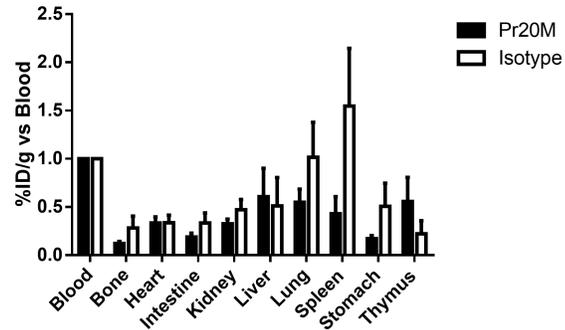


β -half-life = approx 4.5 days

B

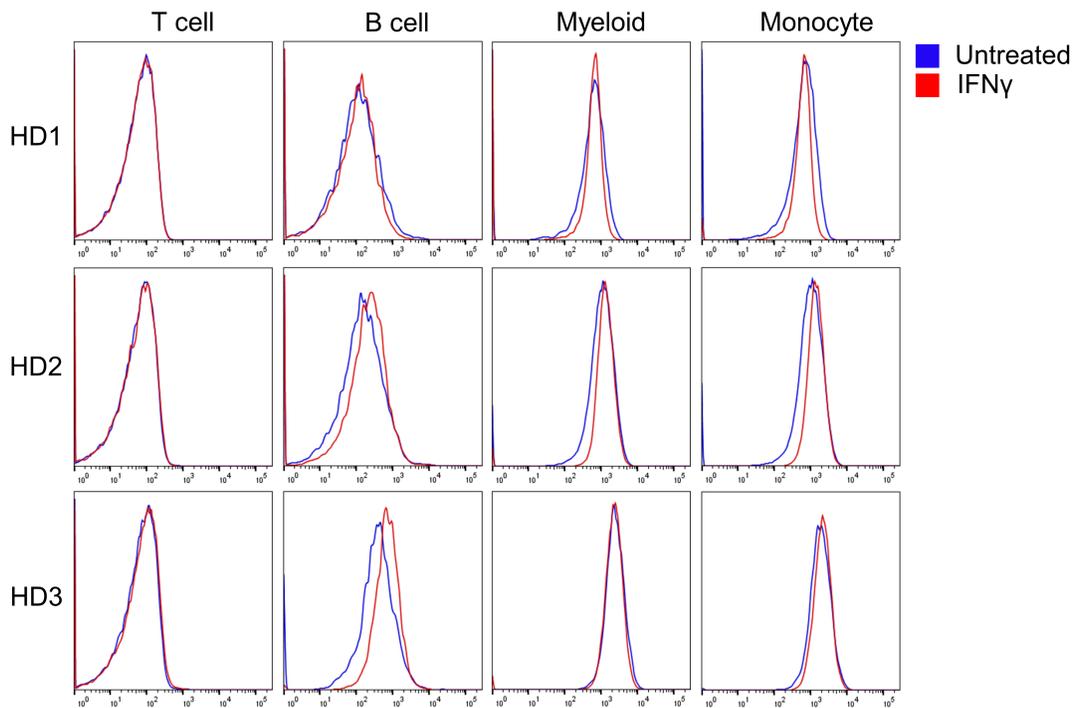
HLA-A2 Transgenic Mice

Pr20M Biodistribution - Normalized to Blood



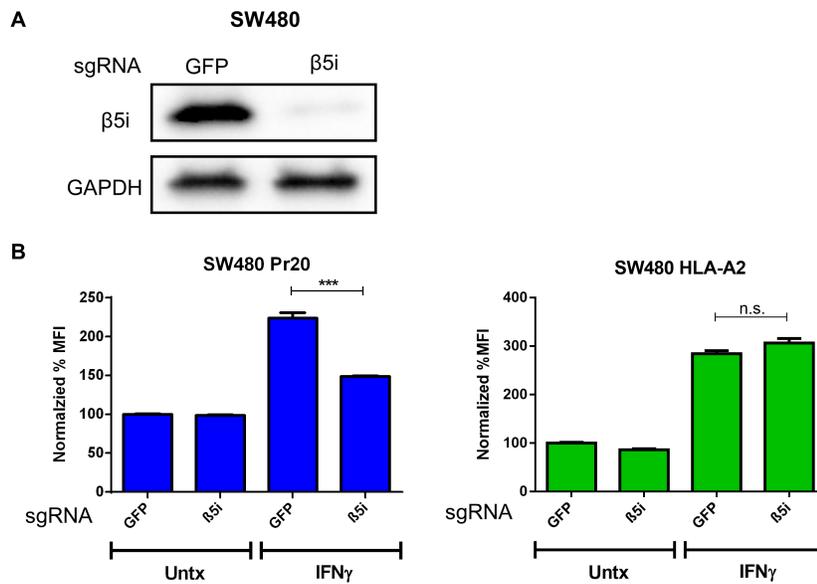
Supplemental Figure 3. Pr20M demonstrates favorable blood pharmacokinetics and does not obviously accumulate in major organs *in vivo*.

Pr20M was radiolabeled with ^{125}I . (A) WT C57BL/6J mice ($n=3$) were injected retro-orbitally with trace amounts ($2.5\ \mu\text{g}$) of radiolabeled Pr20M. Peripheral blood was collected at the indicated time points, scintillation counting was used to determine amount of Pr20M, and % initial dose (ID) / mL is graphed. Experiment was performed once. (B) HLA-A2 transgenic mice were injected retro-orbitally with $2.5\ \mu\text{g}$ of radiolabeled Pr20M ($n=3$ per treatment group). 24 hours later, indicated organs were harvested and the % injected dose/g normalized to each mouse's % injected dose/g in blood is graphed. Experiment was performed once.



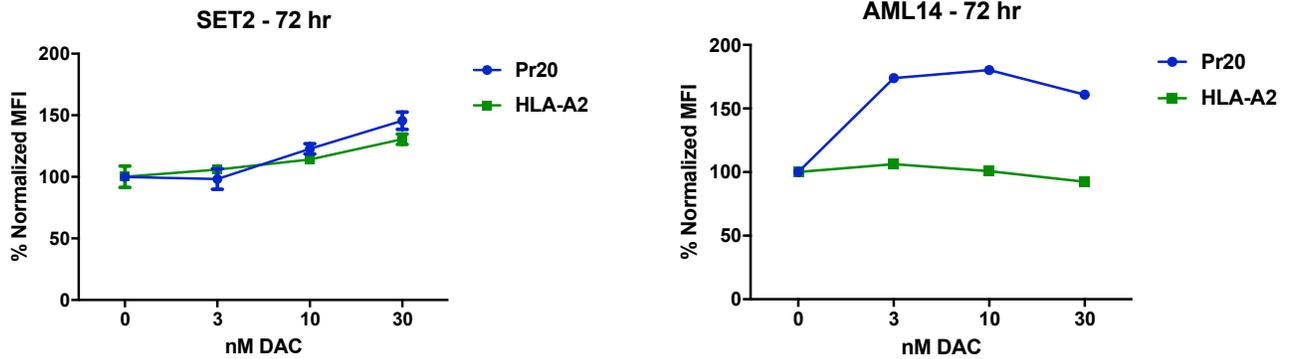
Supplemental Figure 4. IFN γ does not induce substantial Pr20 binding in HLA-A2+ healthy donor PBMC populations

HLA-A2+ healthy donor (HD) PBMC were left untreated or incubated with 10 ng/mL IFN γ for 72 hours. Flow cytometry was used to determine Pr20 binding in major PBMC populations (T, B, myeloid, and monocyte). Gating strategy was performed as in figure 2B. The experiment was performed on PBMC from n=3 HLA-A2+ healthy donors.



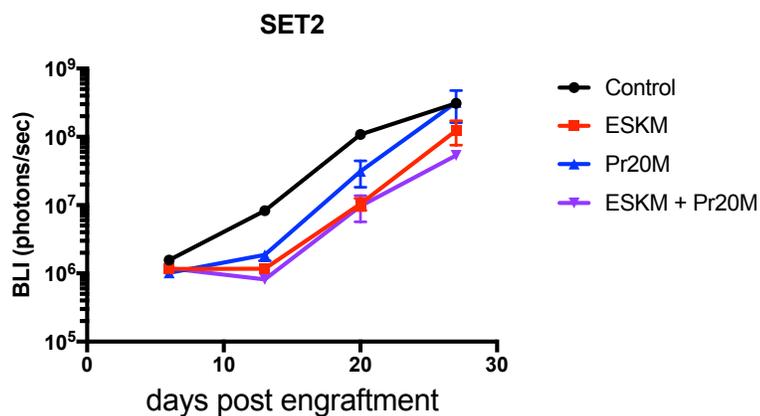
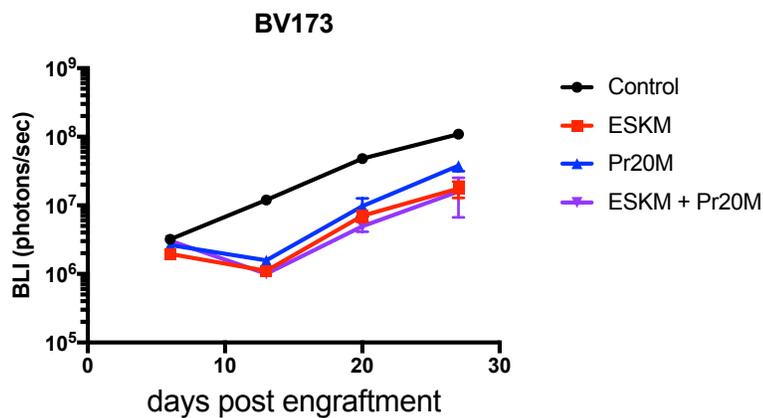
Supplemental Figure 5. $\beta 5i$ knock-out by CRISPR abrogates IFN γ -mediated Pr20 binding in colon adenocarcinoma SW480.

CRISPR constructs against the immunoproteasome subunit $\beta 5i$ or the irrelevant GFP was generated and transduced as described in figure 4. (A) Western blot analysis was used to confirm successful knock-out. (B) SW480 cells transduced with the CRISPR construct against $\beta 5i$ or GFP were treated with 10 ng/mL IFN γ for 72 hours and Pr20 binding and surface HLA-A2 expression was measured by flow cytometry. Normalized %MFI is plotted with and without treatment. Experiment was performed 3 times with 3 technical replicates per experiment. Mean \pm SEM are plotted from one representative experiment. Differenced analyzed by unpaired T test.



Supplemental Figure 6. Decitabine increases Pr20 binding *in vitro*

SET2 and AML14 cells were treated with the indicated dose of decitabine or DMSO vehicle for 72 hrs. Due to decitabine instability at 37° C, media with decitabine was replaced daily. Flow cytometry was used to determine MFI of Pr20 and HLA-A2 relative to DMSO vehicle control. Experiments were performed twice with SET2 and once with AML14.



Supplemental Figure 7. Pr20M and an additional TCRm (ESKM) are not additive in TCRm therapy in vivo

NSG mice were engrafted with 0.5×10^6 SET2 cells (AML) or 3×10^6 BV173 cells (ALL) through tail vein injection. On day 6, mice were randomized into groups of 5. Mice were treated on day 6, 10, 13, and 17 after engraftment with 50 μ g of Pr20M alone, 50 μ g of ESKM (a TCRm against a peptide from WT1) alone, or Pr20M and ESKM in combination. Control mice were left untreated and tumor burden was assessed by bioluminescent imaging on the indicated days. Mean group BLI \pm SEM is shown. BLI data was not significantly different on day 20 or 29 for either SET2 or BV173 models between the the ESKM + Pr20M combination group versus the ESKM alone or Pr20M alone (unpaired t-test). Experiment was performed once in the BV173 model and once in the SET2 model.