

PD-L1 shark nanobody-based CAR-T cells

A novel PD-L1-targeted shark V_{NAR} single domain-based CAR-T strategy for treating breast cancer and liver cancer

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14 **Running title:** Shark V_{NAR} single domain-based CAR-T targeting PD-L1

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15 **Abstract**

16 Chimeric antigen receptor (CAR)-T cell therapy shows great potency against hematological malignancies,
17 whereas it remains difficult to treat solid tumors mainly due to lack of appropriate antigenic targets and
18 immunosuppressive tumor microenvironment (TME). Checkpoint molecule PD-L1 is widely
19 overexpressed on multiple tumor types, and the PD-1/PD-L1 interaction is a key mediator of
20 immunosuppression in TME. Here, we isolated anti-PD-L1 single domain antibodies from a newly
21 constructed semi-synthetic nurse shark V_{NAR} phage library. We found that one V_{NAR} , B2, showed cross-
22 reactivity to human, mouse, and canine PD-L1 antigens, and it partially blocked the interaction of human
23 PD-1 to PD-L1. Furthermore, CAR (B2) T cells specifically lysed human breast cancer and liver cancer
24 cells by targeting constitutive and inducible expression of PD-L1, and also hindered tumor metastasis.
25 Importantly, the combination of CAR (B2) T cells with CAR-T cells targeting liver cancer-specific
26 antigen GPC3 regress liver tumors in mice. We concluded that PD-L1-targeted shark V_{NAR} single domain-
27 based CAR-T therapy is a novel strategy to treat breast cancer and liver cancer. This provides a rationale
28 for potential use of CAR (B2) T cells as a monotherapy or combination with a tumor-specific therapy in
29 clinical studies.

30

31 **Keywords:** shark V_{NAR} , single domain antibody, CAR-T cells, immune checkpoint, PD-L1, triple-
32 negative breast cancer, hepatocellular carcinoma or HCC, liver cancer, glypican-3 or GPC3, xenograft.

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33 **Background:**

34 Adoptive cell therapy (ACT), particularly chimeric antigen receptor (CAR)-T cell therapy, has shown
35 great potency as one of the most effective cancer immunotherapies¹⁻³. CARs are synthetic receptors
36 consisting of an extracellular domain, a hinge region, a transmembrane domain, and intracellular signal
37 domains (e.g. CD3-zeta, CD28, 41BB) that initiate T cell activation⁴⁻⁶. CARs can promote non-major
38 histocompatibility complex (MHC)-restricted recognition of cell surface components, bind tumor antigens
39 directly, and trigger a T-cell anti-tumor response⁷. CAR-T cells targeting B cell antigen CD19 have
40 shown clinical success in patients with advanced B cell lymphoma, which led to their approval by the U.S.
41 Food and Drug Administration (FDA)^{3,8}. However, the translation of CAR-T cells to solid tumors is more
42 difficult because of a lack of appropriate antigenic targets and the complex immunosuppressive tumor
43 microenvironment (TME). Recently, the proteins glypican-2 (GPC2)⁹, glypican-3 (GPC3)¹⁰, and
44 mesothelin^{11,12} were reported as emerging antigens for CAR-T therapy in the treatment of solid tumors
45 and development for clinical trials. However, not all tumors express highly specific surface antigens that
46 are suitable for CARs recognition. Tumor heterogeneity makes targeted therapy more challenging.
47 Programmed death-ligand 1 (PD-L1 or CD274) has aberrantly high expression on multiple tumor types
48 through oncogenic signaling¹³, and is induced by pro-inflammatory factors such as IFN- γ in the immune-
49 reactive TME¹⁴. It has been shown that PD-L1 expressed on tumors can induce T-cell tolerance and avoid
50 immune destruction through binding with its ligand programmed cell death protein 1 (PD-1) on T-cell,
51 which may be one of the main reasons for the poor effect of CAR-T in solid tumors¹⁵. Clinically,
52 antibody-based PD-1/PD-L1 antagonists were reported to induce durable tumor inhibition, especially in
53 melanoma, non-small cell lung cancer, and renal cancer. However, the response rate remains poor in other
54 types of advanced solid tumor¹⁶. Recently, PD-L1-targeting camelid V_HH-nanobody-based CAR-T cells
55 have shown to delay tumor growth in a syngeneic mouse melanoma model¹⁷. Moreover, PD-L1-targeting
56 CAR natural killer (NK) cells inhibited the growth of triple-negative breast cancer (TNBC), lung cancer,
57 and bladder tumors engrafted in NOD SCID gamma (NSG) mice¹⁸. Furthermore, bi-specific Trop2/PD-
58 L1 CAR-T cells targeting both Trop2 and PD-L1 demonstrated improved killing effect of CAR-T cells in

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59 gastric cancer¹⁹. PD-L1-targeted CAR-T cell therapy is presumed to kill PD-L1-overexpressing tumor
60 cells and block the PD-1/PD-L1 immune checkpoint, thereby significantly enhancing anti-tumor activity
61 in solid tumors.

62 The single-chain antibody variable fragment (scFv) commonly serves as the antigen-recognition region of
63 a CAR construct, which consists of heavy (V_H) and variable light (V_L) chains connected by a flexible
64 linker (Gly_4Ser)₃. However, folding of an artificially engineered scFv can affect the specificity and
65 affinity of the CAR for its target antigen²⁰. In contrast, the antigen binding domain of naturally occurring
66 single-domain antibodies (heavy chain-only) from camelid (V_{HH})²¹ and shark (V_{NAR})²² have beneficial
67 properties for the engineering of CARs. They are small in size (12-15 kDa), easily expressed, and capable
68 of binding concave and hidden epitopes that are not accessible to conventional antibodies²³. Remarkably,
69 shark V_{NARS} have unique features that are distinct from camel V_{HH} s—they are in large diversity, and are
70 evolutionally derived from an ancient single domain that functions as a variable domain in both B cell and
71 T cell receptors^{24,25}. We previously constructed a V_{NAR} phage-displayed library from six nurse sharks²⁶.
72 Currently, there are several shark V_{NARS} emerging from pre-clinical research. Their therapeutic and
73 biotechnological applications are under intensive investigation²⁷⁻²⁹.

74 In this study, we reconstructed a semi-synthetic shark V_{NAR} phage library with randomized third
75 complementarity-determining regions (CDR3) of 18 amino acids (AA) in length. Of the three binders that
76 were cross-reactive with mouse and human antigens, only B2 could functionally block the interaction
77 between human PD-L1 and PD-1. More importantly, B2-based CAR-T cells successfully inhibited tumor
78 growth in the xenograft mouse models of TNBC and hepatocellular carcinoma (HCC). Interestingly, the
79 combination of CAR (B2) T cells and liver cancer specific GPC3 CAR demonstrated better efficacy in a
80 synergistic manner compared to single antigen-targeted CAR-T cells in mice, highlighting the feasibility
81 and efficacy of PD-L1-targeting shark V_{NAR} -CAR-T cells in solid tumors.

82 **Results:**

83 **Construction of a semi-synthetic shark V_{NAR} single domain library**

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84 We previously constructed a naïve shark V_{NAR} library from 6 naïve adult nurse sharks (*Ginglymostoma*
85 *cirratum*) with a size of 1.2×10^{10} pfu/ml^{25,26}. To improve the diversity and utility of the shark V_{NAR}
86 library, in this study we developed a semi-synthetic randomized CDR3 18AA shark V_{NAR} library (referred
87 to as ‘18AA CDR3 shark library’). As illustrated in Fig. 1A, 70% of V_{NARS} in the naïve nurse shark
88 library are type II, containing two canonical cysteines located at amino acid 21 and 82 to form a disulfide
89 bond and at least one extra cysteine in CDR1 and CDR3 to form an interloop disulfide bond. Since the
90 type IV V_{NAR} sequence is the closest to its mammalian counterpart such as human V_{H} with only a pair of
91 canonical cysteines, one before CDR1 and the other before CDR3, we made the C29Y mutation and
92 randomized CDR3 loop region to change all V_{NARS} to type IV instead of four types (type I, II, III, and IV).
93 The diversity of the newly semi-synthetic library is 1.2×10^{10} pfu/ml which is comparable with the naïve
94 shark V_{NAR} library (Fig. 1A and 1B). To assess the randomness of sequence modification, we estimated
95 the average nucleotide ratio at each CDR3 residue based on sequencing analysis and found that the CDR3
96 nucleotides were completely randomized with desired ATGC bases ratios (Fig. 1C).

97 **Isolation of cross-species V_{NAR} single domains with high affinity for PD-L1**

98 To identify the anti-PD-L1 shark V_{NAR} that can play a role in the murine tumor environment, we used
99 mouse PD-L1 (mPD-L1) protein as an antigen to screen the new semi-synthetic shark library (Fig. 1A).
100 After four rounds of panning, $\approx 1,000$ -fold enrichment of eluted phage colonies was obtained (Fig. 1D).
101 We also observed an enhanced binding to PD-L1 after the first round of phage panning (Fig. 1E). At the
102 end of the fourth round of panning, 46 individual clones were identified to bind mPD-L1 protein by the
103 monoclonal phage enzyme-linked immunosorbent assay (ELISA), and 11 unique binders were confirmed
104 by subsequent sequencing. Three PD-L1-specific V_{NARS} , B2, A11, and F5, finally showed cross-reactivity
105 to both mouse (mPD-L1) and human PD-L1 (hPD-L1) protein in either His-tag or the hFc-tag formats, as
106 shown by monoclonal phage ELISA (Fig. 1F-H).

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107 To determine the antigen specificity of shark V_{NARS} , we established three PD-L1 knockout (KO) single
108 clones by the CRISPR-Cas9 technology in a human TNBC cell line, MDA-MB-231. To enhance the PD-
109 L1 knockout efficiency, two single guide RNAs (sgRNAs) were designed to target the promoter of the
110 endogenous PD-L1 gene (Fig. 2A). All three individual cell clones confirmed the loss of PD-L1
111 expression (Fig. 2A), and clone 1 was further used in the present study. To determine cross-species
112 reactivity of anti-PD-L1 shark V_{NARS} against native PD-L1, three PD-L1 positive tumor cell lines,
113 including a human breast cancer cell line, a mouse melanoma cell line, and a canine melanoma cell line,
114 were used to evaluate binding activity of B2, A11, and F5. As shown in Fig. 2B, both B2 and F5 bind
115 human antigen, and cross-react with mouse and canine antigens. B2 showed a higher binding ability to
116 both human and mouse antigens than that of F5. A11 bind canine antigen but not antigen of human or
117 mouse. In contrast, no binding was shown on PD-L1 KO cells, indicating the binding activity of shark
118 V_{NARS} is antigen-specific. To determine binding kinetics, we further produced V_{NAR} -Fc fusion protein and
119 incubated them with hPD-L1-His protein on the Bio-layer interferometry (BLI) Octet platform. The K_D
120 value of the B2 was 1.7 nM and 1.4 nM at a concentration of 100 nM and 50 nM respectively, whereas F5
121 failed to bind hPD-L1 protein on Octet (Fig. 2C). To further examine whether B2 was able to functionally
122 block the interaction between human PD-1 (hPD-1) and hPD-L1, we developed a blocking assay based on
123 BLI technology. As shown in Fig. 2D, B2 partially blocked the interaction of hPD-1 to hPD-L1 compared
124 with both F5 and PBS control. Moreover, B2 showed specific binding to hPD-L1 but not human B7-H3,
125 which is another B7-CD28 family member (Fig. 2E).

126 To explore the binding epitope of anti-PD-L1 nanobodies, we synthesized a peptides array based on hPD-
127 L1 extracellular domain (ECD) that consists of total 24 peptides. As shown in Fig. S1 and 2F, both F5
128 and B2 significantly bind to the same peptide #19 (TTNSKREEKLFNVTSTLR), while A11 did not bind
129 to any peptides. In comparison with F5, B2 showed specific binding to peptide #4
130 (TIECKFPVEKQLDLAALI), which overlaps with the PD1/PD-L1 binding site on the final amino acid
131 "T".

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132 Altogether, we have successfully identified functionally cross-species anti-PD-L1 shark single-domain
133 antibodies with high affinity.

134 **PD-L1 (B2) CAR-T cells kill breast cancer cells**

135 Flow cytometric analysis showed that PD-L1 was highly expressed in multiple human tumor types,
136 including breast cancer (MDA-MB-231), ovarian cancer (IGROV-1, OVCAR8, and NCI-ADR-RES),
137 pancreatic cancer (KLM1 and SU8686), and lung cancer (EKVX), suggesting that PD-L1 is a putative
138 pan-cancer antigen (Fig. 3A). To determine whether our shark V_{NARS} can be used for the CAR-T
139 therapeutic approach, we constructed CARs containing the B2 V_{NAR} as the antigen recognition region,
140 along with 4-1BB, CD3 ζ signaling domains, and a truncated human EGFR cassette to gauge transduction
141 efficiency and to switch CAR off (Fig. 3B). The transduction efficiency of V_{NAR} based CAR T cells was
142 high (~90%) (Fig. 3C). During days 7–12, non-transduced mock T cells and CAR (B2) T cells showed
143 indistinguishable expression of exhaustion markers (PD-1 and TIM-3) compared with each other, whereas
144 slightly higher expression of LAG-3 was found in CAR (B2) T than mock T cells (Fig. 3D). MDA-MB-
145 231 is a highly aggressive, invasive, and poorly differentiated TNBC cell line with limited treatment
146 options. We, therefore, used it as a tumor model by engineering it to overexpress GFP/Luciferase (GL)
147 for a luciferase-based cytolytic assay. Both mock T and CAR (B2) T cells were incubated with MDA-
148 MB-231 GL cells for 24 hours or 96 hours. As shown in Fig. 3E, tumor cells were effectively lysed by
149 CAR (B2) T cells in a 2-fold dose-dependent manner compared with mock T cells. Moreover, the long
150 incubation time of 96-hours could efficiently increase the cytotoxicity of CAR (B2) T cells even at the
151 lowest Effector: Target (E/T) ratio of 1:3. To investigate whether the cytolytic activity of CAR (B2) T
152 cells is antigen-dependent, we incubated CAR (B2) cells with its corresponding PD-L1 KO cell line,
153 showing that CAR T cells were not capable of killing antigen KO cells (Fig. 3E). A significantly higher
154 level of TNF- α , IL-2, and IFN- γ was released from CAR T cells when co-cultured with tumor cells at 5:1
155 or 2.5:1 E/T ratios, while minimum cytokine production was observed from mock T cells (Fig. 3F). These

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156 results suggested that V_{NAR} -derived T cells were able to efficiently lyse tumor cells. Furthermore, we
157 included a corresponding soluble B2 V_{NAR} in the co-culture setup to detect whether it could affect the
158 cytotoxicity of CAR (B2) T via blocking the recognition site on tumor cells competitively. As shown in
159 Fig. 3G, inclusion of the B2 single domain significantly inhibited the cytolytic activity of CAR (B2) T
160 cells. In contrast, no specific lysis in tumor cells was found in either coincubation with mock T cells or
161 tumor cells alone in the presence of B2. Taken together, we concluded that CAR (B2) T cells could
162 specifically lyse PD-L1 positive human tumor cells.

163 **CAR (B2) T cells inhibit orthotopic breast cancer in mice**

164 To evaluate anti-tumor efficacy of CAR (B2) T cells in mice, we established an orthotopic breast tumor
165 xenograft model via implanting the MDA-MB-231 GL line into the fourth mouse mammary fat pad.
166 Seventeen days after tumor inoculation, mice were intravenously (IV) infused with either CAR (B2) T
167 cells or antigen-mismatched CAR (CD19) T cells (Fig. 4A). We used both bioluminescence intensity and
168 tumor volume to track the antitumor efficacy of CAR T cells. Mice were followed up to 8 weeks post
169 CAR-T cell infusion except three mice from the control CAR (CD19) group or CAR (B2) treatment
170 group that were euthanized at week 3. As shown in Fig. 4B and 4C, CAR (B2) T cells dramatically
171 reduced breast tumor burden without a marked loss of body weight (Fig. 6D). Importantly, after 5 weeks
172 of CAR-T infusion, we found that tumors metastasized in the control CAR (CD19) group (Fig. 4B and
173 4E). In contrast, no tumors metastases were found in the liver or lungs of mice that were treated with
174 CAR (B2) T cells (Fig. 4B and 4E), indicating that CAR (B2) T cells were able to treat metastatic lesions.
175 To determine CAR-T persistence, we recovered both CAR (CD19) and CAR (B2) T cells from mouse
176 spleen. We found that *ex vivo* CAR (B2) T cells recovered from mice had a comparable persistence after
177 3 weeks infusion (Fig. 4F). Importantly, these spleen-isolated CAR (B2) T cells still showed significant
178 *ex vivo* cytotoxicity against PD-L1 positive tumor cells compared to KO cells (Fig. 4G).

179 **CAR (B2) T cells kill liver cancer cells by targeting inducible expression of PD-L1**

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180 Inducible but not the constitutive expression of PD-L1 can be found in liver cancer cell line Hep3B upon
181 co-incubation with CAR (B2) T cells (Fig. 5A) possibly as a consequence of massive IFN- γ released from
182 co-cultured CAR (B2) T (Fig. 5B). To test anti-tumor effect of CAR (B2) T to mimic the suppressive
183 TME, we established the Hep3B xenograft mouse model with intraperitoneal (IP) injection of Hep3B GL
184 tumor cells. After 12 days of tumor inoculation, mice were infused IP with CAR-T cells (Fig. 5C). We
185 found that four out of five CAR (B2) T mice showed a significant decrease in tumor growth compared
186 with the control CAR (CD19) T group after 3 weeks of infusion (Fig. 4D and E). Based on this
187 observation, we think that CAR (B2) T cells might provide a benefit in liver cancer therapy.

188 **CAR (B2) T cells improve the killing effect of CAR (GPC3) T cells in liver cancer**

189 In our previous study, we developed GPC3-targeted CAR-T cells as an emerging liver cancer therapy¹⁰.
190 We observed that CAR (GPC3) T cells killed Hep3B tumor cells efficiently but upregulated PD-L1
191 expression was found in CAR (GPC3) T-cocultured Hep3B cells (Fig. 5F), which may allow cancers to
192 evade the host immune system. Therefore, we hypothesized that the elimination of inducible PD-L1
193 positive tumor cells by CAR (B2) T cells will improve the anti-tumor activity. To detect our hypothesis,
194 we designed two strategies, including bispecific expression or combination of PD-L1 and GPC3 CAR-T
195 cells (Fig. 5G). Bispecific CAR-T cell was produced by co-transducing with GPC3 CAR and CAR (B2)
196 lentivirus (Fig. 5G). To compare their anti-tumor effect, all seven groups of CAR-T cells and mock T
197 cells (Fig. 5G) were incubated with Hep3B cells for 24 hours and 72 hours. As shown in Fig. 5H, the
198 cytotoxicity of bispecific CARs was significantly higher than either of the monospecific CARs, especially
199 at 72 hours of incubation time. Moreover, CAR (B2) T cells can improve the efficiency of CAR (GPC3)
200 T cells in a dose-dependent manner (MOI 2.5 vs 5). Furthermore, we observed higher levels of TNF- α ,
201 IL-2, and IFN- γ were secreted from both bispecific and combination CAR treatment than those of
202 monospecific CAR-T treatments (Fig. 5I). Therefore, we concluded that the bispecific CAR-T and the

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203 combined CAR-T strategies significantly improved the activity of CAR-T cell in liver cancer by targeting
204 both PD-L1 and GPC3.

205 **Combination of CAR (B2) T and CAR (GPC3) T achieves a synergistic anti-tumor effect in mice**

206 To further analyze the functions of bi-specific CAR-T and combination CAR-T strategies in response to
207 liver cancer, we confirmed the anti-tumor effect through the Hep3B xenograft mouse model. Mice
208 bearing Hep3B tumors were divided into five groups and infused with 5 million equivalents of CAR
209 (GPC3) T , CAR (CD19) T, CAR (B2) T, Bi-GPC3/B2 CAR T, and a combination of 2.5 million CAR
210 (GPC3) T and 2.5 million CAR (B2) T (referred to as “Combo-GPC3/B2”) cells, respectively. Tumor
211 luciferase signal was evaluated by bioluminescence imaging weekly, and T cells isolated from week 2
212 mouse blood were analyzed (Fig. 6A). In comparison with the control CAR (CD19) T cells, CAR (GPC3)
213 T and CAR (B2) T cells individually inhibited tumor growth in xenografts (Fig. 6B and 6C). Surprisingly,
214 bispecific CAR-T cells failed to regress tumor burden and the effect was worse compared to monospecific
215 CAR-T cells. However, the combination group showed a significant synergistic anti-tumor effect in
216 xenografts (Fig. 6B and 6C). We sacrificed mice by the end of week 4 after treatment due to maximum
217 tumor limitation. To visualize tumor size, we isolated tumors from a mouse (#1) from combination group
218 and a mouse (#2) from bispecific CAR-T group. As shown in Fig. 6D, the tumor size from combination
219 group was much smaller than that from bispecific CAR treatment group. To identify factors that
220 contribute to the high efficiency in combination CAR-T strategy, we detected number, immunophenotype,
221 and exhaustion of CAR-T cells isolated from mouse blood at week 2 of infusion. We found that mice
222 receiving CAR (B2) T, Combo-GPC3/B2 CAR T, or Bi-GPC3/B2 CAR T cells had much higher
223 CD3+CAR+ T cells counts in blood than those who received CAR (CD19) T or CAR (GPC3) T cells
224 (Fig. 6E). On the other hand, the number of CAR (B2) T was higher than that of combination followed by
225 bispecific CAR-T cells, indicating bispecific CAR-T might loss PD-L1-specific proliferation. Indeed, we
226 found that the recovered CAR-T cells from Combo-GPC3/B2 mouse (#143) showed a higher binding
227 ability to PD-L1 than the CAR-T cells of Bi-GPC3/B2 mouse (#107) (21.2% vs 16.4%) though both

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228 CAR-T groups showed similar binding percentage in cell culture (3.28% vs 2.62%) (Fig. 6F). Moreover,
229 the CAR-T cells recovered from the mouse spleen showed higher binding ability compared with *in vitro*
230 cultured CAR-T cells, especially on CAR (B2) T cells (5.93% vs 35.4%). Besides the functional capacity
231 of endogenous T cells, the frequency of memory T cell subset is also associated with tumor response.
232 Here, we analyzed the T differentiation subsets consisting of stem cell-like memory T cells (T_{SCM} :
233 CD62L+CD45RA+CD95+), central memory T cells (T_{CM} : CD62L+CD45RA-CD95+), effector memory
234 T cells (T_{EM} : CD62L-CD45RA-CD95+), and terminally differentiated effector memory T cells (T_{EMRA} :
235 CD62L-CD45RA+CD95+) in CD4+CAR+ and CD8+CAR+ subpopulations in mouse blood after 2
236 weeks of infusion. As shown in Fig. 6G, the combination group exhibited a significantly higher
237 percentage of Tscm than CAR (B2) T and higher frequency of Tem and Tcm than CAR (GPC3) T on
238 both CD4+ and CD8+ subpopulations. We further analyzed the expression of co-inhibitory receptors in
239 CAR-T cells, including PD-1, LAG-3, and TIM-3. The CAR-T cells that containing B2 showed higher
240 expression of PD-1 and LAG-3 than CAR (GPC3) in both CD4+ and CD8+ subpopulations (Fig. 6H).
241 Collectively, these results suggest that a combination of CAR (GPC3) T and CAR (B2) T, but not
242 bispecific CAR, synergistically killed Hep3B tumor.

243 Discussion

244 Checkpoint molecule PD-L1 is highly expressed on many tumors in a constitutive or IFN- γ -inducible
245 manner. IFN- γ is the key functional cytokine released from effector T cells; however, the increased
246 expression of PD-L1 on tumor cells binding to PD-1 on effector T cells results in T cell exhaustion, and
247 inhibition of T cell functions³⁰. In this study, we hypothesized that the development of CAR-T cells
248 targeting PD-L1 could kill solid tumors via recognizing the constitutive or inducible expression of PD-L1
249 in the tumor immunosuppressive microenvironment. To test our hypothesis, we isolated a panel of anti-
250 PD-L1 single domain antibodies from a newly established semi-synthetic nurse shark V_{NAR} library. The
251 best candidate, B2, showed a specific binding ability to PD-L1, and was cross-reacting with both human
252 and mouse antigens. Importantly, B2 functionally blocked the interaction between PD-L1 and PD-1.

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253 Moreover, we found that single domain-based CAR-T showed much higher transduction efficiency than
254 scFv-based CAR-T cells, indicating that single domain antibodies are more appropriate to be engineered
255 into CAR format because it is smaller, easily expressible, and more stable.

256 PD-L1 is not only overexpressed on a larger number of malignancies, but also on immune cells in the
257 tumor microenvironment¹³. T cells express low levels of endogenous PD-L1, which leads to the
258 development of CAR-T cells that targeting PD-L1 is somewhat intricate by killing PD-L1 expressing
259 tumor cells and blocking the PD-1/PD-L1 checkpoint axis^{31,32}. Antigen exposure of CAR-T cells may
260 lead to T cell fratricide and exhaustion, impairing the proliferation and persistence of CAR-T cells *in vitro*
261 and *in vivo*. Xie *et al.* reported that camelid V_HH-based anti-mouse PD-L1 CAR-T cells can “self”-
262 activate *in vitro* and PD-L1 deficient CAR-T cells could live longer than WT CAR-T³¹. However, during
263 a period of 7-12 days *in vitro* co-culturing with CD3/CD28 microbeads, we did not find upregulation in
264 PD-L1 (Fig. S2) or exhaustion markers (PD-1, TIM-3, and LAG-3) in the activated CAR (B2) T cells
265 compared with mock T cells *in vitro*. These events were probably due to PD-L1 antigen endocytosis
266 caused by anti-PD-L1 CAR-T cells themselves^{33,34}. Interestingly, we did not observe any cytolytic
267 phenomenon or upregulated IFN- γ expression in the cultured CAR (B2) T cells, indicating that the
268 cytotoxicity of CAR (B2) T was not triggered by the T cells’ endogenous PD-L1. We consider that less
269 tonic signaling of our shark V_{NAR}-based CAR (B2) T cells may be due to the relative low binding affinity
270 of CAR (B2). Ghorashian *et al.* reported the enhanced proliferation and anti-tumor activity in a lower
271 affinity CD19 CAR comparing with that in a clinical high affinity CD19 CAR-T, indicating that the
272 increased immunoreceptor affinity may adversely affect T cell responses³⁵.

273 To overcome tumor escape mechanisms and enhance the anti-tumor effect of CAR-T cells, a combination
274 strategy might be more feasible in solid tumor therapy, such as combining CAR-T cells with monoclonal
275 antibodies, small-molecules, or bi-specific CAR T cells targeting different tumor-specific antigens^{36,37}. In
276 our study, we found that CAR (B2) T cells could kill liver cancer cells by targeting inducible PD-L1 in
277 the immunosuppressive TME (Fig. 5D), whereas B2 V_{NAR} did not show a significant benefit in improving
278 cytotoxicity of CAR (GPC3) T cell even though it functionally blocked the interaction of PD-1 to PD-L1

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279 (Fig. S3). Thus, we constructed the bispecific CAR-T cell targeting both HCC tumor-specific antigen
280 GPC3 and inducible tumor-immunosuppressive antigen PD-L1. Surprisingly, Bi-GPC3/B2 CAR T cells
281 worked best *in vitro* whereas only slightly inhibited liver tumor progression *in vivo*, and even worse than
282 the individual CAR (GPC3) T and CAR (B2) T cells. We think it may be due to low CAR density or low
283 binding affinity when we co-transduced B2 and GPC3 CAR lentivirus into PBMCs. In future work, we
284 may optimize the bi-specific CAR construct by optimizing GPC3 and B2 CAR fragments into one
285 construct¹⁹. Encouragingly, the combination of CAR (GPC3) T and CAR (B2) T cell achieved a
286 synergistic anti-tumor effect *in vivo*. A previous study reported that the combination of anti-mesothelin
287 CAR-T cell with anti-PD-L1 CAR-T did not repress tumor growth synergistically in PDX, as anti-PD-L1
288 CAR-T killed anti-mesothelin CAR-T cell by targeting its endogenous PD-L1 antigen³³. In our study, we
289 did not observe upregulated PD-L1 expression in CAR (GPC3) T cells probably due to different CAR
290 constructs. On the other hand, we found that the expansion of CAR-T count in mouse blood is highly
291 correlated with the presence of CAR (B2) construct.. It may be due to the cross-recognition of CAR (B2)
292 to mouse antigen, but the CAR-T treatment mice were healthy and did not experience body weight loss,
293 indicating our CAR (B2) T cells are safe for the mouse. Although we didn't observe upregulated PD-1
294 expression in the cultured CAR (B2) T cells, the high expression of PD-1 was found in *ex vivo* B2-related
295 CAR-T cells (Fig. 6G). However, the recovered CAR (B2) T cells from 3 weeks after infusion still
296 efficiently lysed the MDA-MB-231 cells (Fig. 6G), probably due to the B2 V_{NAR} blocking the interaction
297 of PD-1 to PD-L1 even though not entirely.

298 **Conclusions**

299 We have demonstrated that a semi-synthetic shark V_{NAR} phage library based on fully randomized CDR3
300 can be used in isolating anti-PD-L1 specific single domain antibodies. We conclude that the PD-L1-
301 targeted shark V_{NAR}-based CAR-T cell is a promising strategy in triple-negative breast cancer and liver
302 cancer therapy, providing a rationale for the potential use of PD-L1 (B2) CAR-T cells in clinical studies.
303 Overall, the results in this study demonstrate the feasibility and the efficacy of CAR-T cells targeting

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304 tumor immunosuppressive microenvironment antigen PD-L1 against aggressive solid tumors. To improve
305 treatment of solid tumors, future efforts should be directed at utilizing genome editing to develop “off-
306 the-shelf” fratricide-resistant PD-L1-targeted CAR-T cells lacking both endogenous PD-L1 and T cell
307 receptor alpha chain expression on T cells.

308

309 **Materials and Methods**

310 **Construction of a synthetic 18AA CDR3 nurse shark V_{NAR} phage library**

311 We constructed the new synthetic 18AA CDR3 nurse shark V_{NAR} phage library based on our previous
312 naïve shark library²⁶. For the V_{NARS} DNA cassettes, a non-canonical cysteine in CDR1 was mutated to
313 tyrosine (C29Y) using naïve shark library V_{NARS} pComb3x plasmid as the template. Subsequently, a pair
314 of randomized 18AA CDR3 primers was designed to amplify the CDR3 loop using the PCR method.
315 PCR product were circularized by intra-molecular self-ligation in 1 ml of ligation buffer using T4 DNA
316 ligase (New England Biolabs, Ipswich, MA). Finally, the ligation products were purified by removing the
317 enzymes and transformed into 500 µl of electroporation competent TG1 cells (Lucigen, Middleton, WI) to
318 make the library.

319 **Phage panning**

320 The phage panning protocol has been described previously^{26,38}. The mPD-L1 protein bought from R&D
321 Systems was used for four rounds of panning. Details are provided in the supplemental materials.

322 **Affinity binding and blocking activity**

323 The binding kinetics of the V_{NAR}-hFc (produced by GenScript) to hPD-L1-His protein (SinoBiological)
324 was determined using the Octet RED96 system (FortéBio) at the Biophysics Core (National Heart, Lung
325 and Blood Institute or NHLBI) as described previously³⁹. The blocking activity of B2-hFc was also

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326 determined using the BLI Octet platform as described previously⁴⁰. Details are provided in the
327 supplemental materials.

328 **Generation of anti-PD-L1 nanobody-based CAR-T cells**

329 We generated the PD-L1-target shark V_{NAR}-based CAR-T lentiviral vector following the design principle
330 of CAR construct published in our previous study¹⁰. Briefly, the V_{NAR} fragment of B2 was subcloned into
331 a CAR construct (pMH330). The CAR expressing lentivirus was produced as described previously¹⁰.
332 PBMCs isolated from healthy donors were stimulated for 24h using anti-CD3/anti-CD28 antibody-coated
333 beads (Invitrogen) at a bead: cell ratio of 2:1 according to manufacturer's instructions in the presence of
334 IL-2.

335 ***In vitro* cytotoxicity of CAR-T cells and activation assays**

336 The cytotoxicity of CAR-T cells was determined by a luciferase-based assay. In brief, the luciferase-
337 expressing MDA-MB-231 and Hep3B tumor cells were used to establish a cytolytic assay. The cytotoxicity
338 of PD-L1-target CAR (B2) T cells was detected by co-culturing with MDA-MB-231 GFP-Luc and
339 Hep3B GFP-Luc at various E/T ratios for 24 hours or 96 hours followed by measurement of the luciferase
340 activity using the luciferase assay system (Promega) on Victor (PerkinElmer). The supernatants were
341 collected for TNF- α , IL-2, and IFN- γ detection using ELISA Kit (BD biosciences). In the killing blocking
342 assay of CAR-T cells, varying concentration of soluble B2 protein was added into tumor CAR-T cells
343 incubation for 24 hours and 48 hours.

344 **Animal studies**

345 5-week-old female NOD/SCID/IL-2Rgc^{null} (NSG) mice (NCI Frederick) were housed and treated under
346 the protocol (LMB-059) approved by the Institutional Animal Care and Use Committee at the NIH. A
347 total of 3 million MDA-MB-231-GFP-Luc cells were suspended in the mixture of PBS: Matrigel (BD

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348 Biosciences) at 1:1, and inoculated into the inguinal mammary fat pad to establish the orthotopic MDA-
349 MB-231 model. Peritoneal Hep3B xenograft tumor model was established as previously described¹⁰.
350 Tumor volume was calculated as $\frac{1}{2} (\text{length} \times \text{width}^2)$ and bioluminescent intensity (Xenogen IVIS
351 Lumina). When the average tumor size reached the indicated size, 5 million CAR-T cells were
352 intravenously injected into mice models. *Ex vivo* T cells were isolated from mice spleens using Miltenyi
353 Biotec tumor dissociation kit, and were cultured *in vitro* with 40ng/ul IL-2, IL-7, and IL-21 in the culture
354 media.

355 **Statistical analysis**

356 All experiments were repeated at least three times to ensure reproducibility of results. All statistical
357 analyses were performed using GraphPad Prism, and are presented as mean \pm SEM. Results were analyzed
358 using 2-tailed unpaired Student's t test. A P value of < 0.05 was considered statistically significant.

359 **Acknowledgments**

360 We thank NIH Fellows Editorial Board for editorial assistance. We thank NCI CCR Animal Resource
361 Program/NCI Biological Testing Branch, NCI CCR/Leidos Animal Facility, Drs. Grzegorz Piszczek and
362 Di Wu of the NHLBI Biophysics Core, NCI CCR Flow Cytometry Core Facility for providing assistance
363 in animal support, V_{NAR} single domain antibody kinetics/affinity analysis and cellular staining.

364 **Authors' Contributions**

365 Conception and design, D.L., G.M., and M.H.; development of methodology, D.L., H.J.E., G.M., C.P.D.,
366 and M.H.; acquisition of data, D.L., H.J.E., J.H., T.Y.Z.L.; analysis and interpretation of data, D.L.,
367 H.J.E., J.H., T.Y.Z.L.; writing, D.L. and M.H.; review and/or revision of manuscript, T.Y.Z.L., G.M.,
368 C.P.D., and M.H.; study supervision, M.H. All authors read and approved the final manuscript.

369 **Funding**

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370 This work was supported by the NCI CCR FLEX Program Synergy Award (to G.M. and M.H.) and the
371 NCI CCR FLEX Program Technology Development Award (to M.H.).

372 **Competing interests**

373 M.H., G.M., D.L., H.J.E., and C.P.D. are inventors on US provisional patent application no. 63/208,755,
374 “Cross Species Single Domain Antibodies Targeting PD-L1 For Treating Solid Tumors”. The authors
375 declare no other competing interests.

376 **Ethics approval**

377 All mice were housed and treated under the protocol (LMB-059) approved by the Institutional Animal
378 Care and Use Committee at the NIH.

379 **Provenance and peer review**

380 Not commissioned, externally peer reviewed.

381 **Data availability statement**

382 All data relevant to the study are included in the article or uploaded as online supplemental information.

383

384

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524 **Figure Legends**

525 **Figure 1.** Isolation of anti-PD-L1 single domain antibody by phage display from an engineered semi-
526 synthetic shark V_{NAR} phage library. (A) Circuit of three steps library construction and phage panning. A
527 18AA randomized CDR3 semi-synthetic shark V_{NAR} phage library was constructed by PCR mutation and
528 gene assemble. After 3-5 rounds of phage panning, anti-mPD-L1 V_{NARS} were isolated from the phage
529 library, and further validated by phage ELISA and protein purification technologies. (B) Information
530 regarding newly shark V_{NAR} library compared with pre-synthetic V_{NAR} library. (C) Pie chart of the
531 percentage of average nucleotide (ACTG) ratio at each randomization NNS. (D) Phage-displayed single-
532 domain antibody clones were identified against recombinant mPD-L1-his after four rounds of panning. A
533 gradual increase in phage titers was observed during each round of panning. (E) Polyclonal phage ELISA
534 from the output phage of each round of panning. (F-H) Cross-reactivity of anti-PD-L1 B2 (F), A11 (G),
535 and F5 (H) to mPD-L1 and hPD-L1 protein within His-tag or hFc-tag by monoclonal phage ELISA
536 analysis.

537 **Figure 2.** Verification of specific binding and blocking ability of anti-PD-L1 shark V_{NARS} . (A) Schematic
538 design for constructing PD-L1 KO MDA-MB-231 cell line using CRISPR-Cas9 method. Two sgRNAs
539 were designed to target the promoter of the endogenous PD-L1 gene. Single PD-L1 KO clones were
540 validated by western blot and flow cytometry. (B) The cross-reactive binding of anti-PD-L1 V_{NARS} to
541 native PD-L1 as determined by flow cytometry. Three different tumor cell lines from human, murine, and
542 canine were stained with V_{NARS} . (C) Binding kinetics of V_{NAR} -hFc to hPD-L1 protein. (D) Blocking the
543 activity of V_{NAR} -hFc to the interaction of hPD-L1 and hPD-1 as determined by the Octet platform. (E)
544 Specific binding of B2 to hPD-L1 and hB7-H3. (F) Epitope mapping of individual B2, F5, and A11.
545 Sequence alignment of PD-L1 ECD region of human, murine, and canine. The conserved residues are
546 marked with asterisks (*), the residues with similar properties between variants are marked with colons (:)
547 and the residues with marginally similar properties are marked with periods(.). The main binding residues

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548 of the hPD-L1 identified previously that interact with PD-1 are shaded in magenta. The binding peptides
549 of B2 to hPD-L1 are highlighted in yellow. Values represent mean \pm SEM. **, $P < .01$; ***, $P < .001$;
550 ****, $P < .0001$; ns, not significant.

551 **Figure 3.** PD-L1 specific V_{NAR} -based CAR-T cells exhibit antigen specific cytotoxicity against MDA-
552 MB-231. (A) Surface PD-L1 expression on multiple human tumor types as determined by flow cytometry.
553 (B) Construct of PD-L1 specific B2 V_{NAR} -based CAR-T cell where CAR and hEGFRt are expressed
554 separately by the self-cleaving T2A ribosomal skipping sequence. (C) The transduction efficiency of
555 CAR (B2) in T cells was determined by hEGFRt expression. Non-transduced T cell was the mock control.
556 (D) Exhaustion marker expression on *in vitro* cultured mock T and CAR (B2) T cell populations. (E)
557 Cytolytic activity of CAR (B2) T cells after 24 or 96 hours of incubation with MDA-MB-231 GL or PD-
558 L1 KO MDA-MB-231 GL respectively in a 2-fold dose dependent manner. (F) TNF- α , IL-2, and IFN- γ
559 concentration in the supernatants of killing assay at E/T ratios of 5:1 and 2.5:1 in Fig. 3D as measured by
560 ELISA. (G) Monovalent B2 nanobody specifically inhibited killing of CAR (B2) T cells on MDA-MB-
561 231 cells after 24 hours and 48 hours of incubation. Tumor cells alone or mock T cells incubation in the
562 presence of B2 nanobody were used as the control in this study. Statistical analyses are shown from three
563 independent experiments. Values represent mean \pm SEM. **, $P < .01$; ***, $P < .001$; ****, $P < .0001$; ns,
564 not significant.

565 **Figure 4.** Tumor regression in the orthotopic MDA-MB-231 xenograft mouse model by CAR (B2) T
566 cells infusion. (A) Schema of the MDA-MB-231 orthotopic xenograft NSG model IV infused with 5
567 million CAR (B2) T cells and CAR (CD19) CAR T cells after 17 days of tumor inoculation. (B)
568 Representative bioluminescence image of MDA-MB-231 tumor growth in the orthotopic model. (C)
569 Tumor size of every mouse measured by a digital caliper [$V=1/2(\text{length width}^2)$]. ****, $P < .0001$. (D)
570 Body weight of mice. Values shown represent mean \pm SEM. (E) Representative pictures showing the

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571 restriction of tumor metastasis in CAR (B2) T cell infusion mice. (F) CAR (B2) T cell persistence and (G)
572 *ex vivo* killing on MDA-MB-231 tumor cells after 3 weeks of CAR-T cell infusion.

573 **Figure 5.** CAR (B2) T cells lysed inducible PD-L1 positive Hep3B cells and improved *in vitro* killing as
574 engineered bispecific CAR or combination strategy with CAR-T targeting GPC3. (A) Inducible PD-L1
575 expression in the Hep3B tumor cells after 24 hours incubation with CAR (B2) T at E/T ratio of 1:2. (B)
576 IFN- γ level in the supernatants of incubation CAR (CD19) T or CAR (B2) T cells with Hep3B cell. (C)
577 Schema of the Hep3B xenograft NSG model IP infused with 5 million CAR (B2) T cells and CAR (CD19)
578 T cells after 12 days of tumor inoculation. (D) Representative bioluminescence image of Hep3B tumor
579 growth in the xenograft model. (E) Tumor bioluminescence growth curve. (F) Inducible PD-L1
580 expression in the Hep3B tumor cells alone or in the Hep3B tumor cells after 24-hours incubation with
581 CAR (GPC3) T at E/T ratio of 1:2 or 1:1. (G) Applicable strategy of bispecific CAR-T cells and
582 combination CAR-T cells targeting GPC3 or PD-L1. (H) Cytolytic activity of engineered CAR-T cells on
583 Hep3B cells after 24 hours or 72 hours incubation *in vitro*. (I) TNF- α , IL-2, and IFN- γ concentration in
584 the co-culture supernatant from (H) as measured by ELISA. Values represent mean \pm SEM. **, P < .01;
585 ***, P < .001; ****, P < .0001; ns, not significant.

586 **Figure 6.** Combined CAR (B2) T with CAR (GPC3) T cells achieve a synergistic anti-tumor effect *in*
587 *vivo*. (A) Schema of the Hep3B xenograft NSG model IP infused with equivalent 5 million CAR T cells
588 after 12 days of tumor inoculation. (B) Representative bioluminescence image of Hep3B tumor growth in
589 the xenograft model (C) Tumor bioluminescence growth curve. (D) The sizes of tumors in mice from
590 combination CAR group (#1 mouse) and bispecific group (#2 mouse) at the end of the study. (E)
591 Absolute CAR-T count was detected in mouse peripheral blood after 2 weeks of treatment. Absolute
592 CAR-T concentration (cells/ μ L) \pm SD for all evaluable mice in each treatment group is shown. (F) The
593 binding ability of both *in vitro* and *in vivo* recovered CAR-T cells to PD-L1 antigen using flow cytometry.
594 (G) Relative proportion of stem cell-like memory (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}),

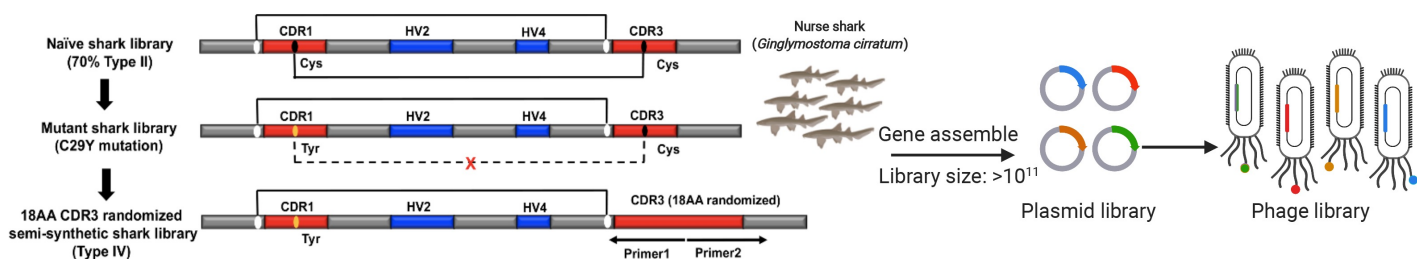
PD-L1 shark nanobody-based CAR-T cells

595 and terminally differentiated effector memory (T_{EMRA}) subsets defined by CD62L, CD45RA and CD95
596 expression in both CD4 + and CD8+ CAR+T cell population in mouse blood on week 2 of treatment. (H)
597 Exhaustion marker expression on CD4 + and CD8 + CAR+T cells populations in mouse blood on week 2
598 of treatment.

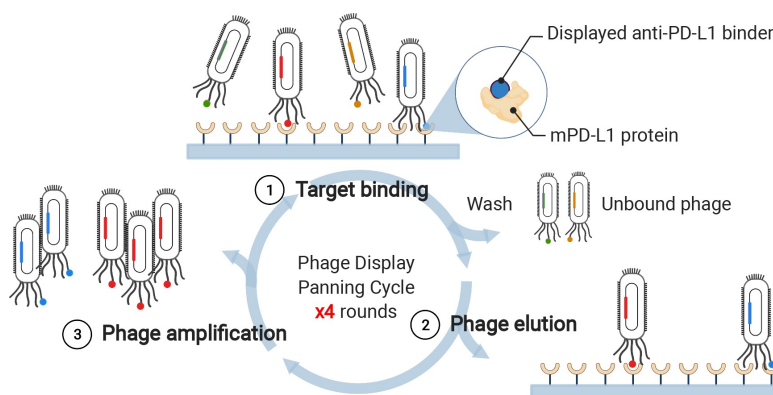
Figure 1

A

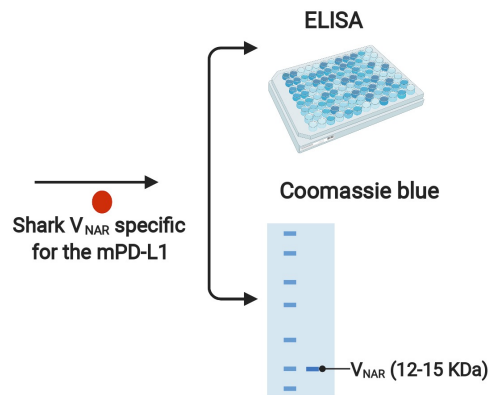
Step 1: Semi-synthetic shark V_{NAR} library construction



Step 2: Phage screening



Step 3: Shark V_{NAR} validation

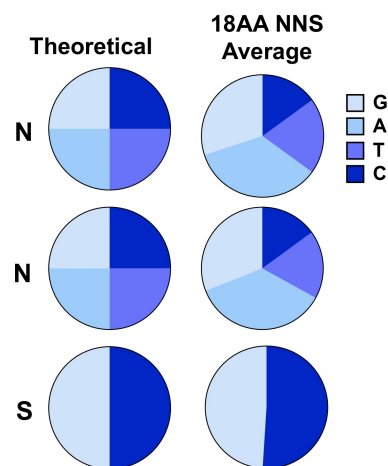


Pick >300 single clones → 46 clones bind PD-L1 by ELISA → 11 unique clone sequences → 3 cross-reactive binders

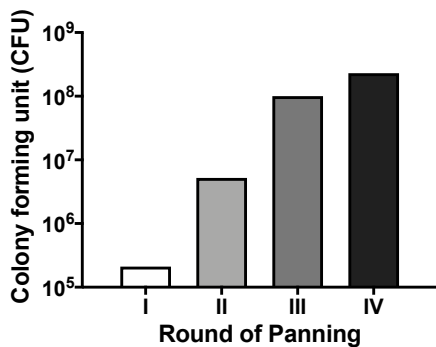
B

Library Name	Naïve or engineered	V _{NAR} types	Diversity (pfu/ml)	CDR3 length
Shark V _{NAR} library	Naïve	Type I, II, III and IV	1.2x10 ¹⁰	0-34 AAs
Shark CDR3 18AA library	CDR3 randomized	Type IV	1.2x10 ¹⁰	18AAs

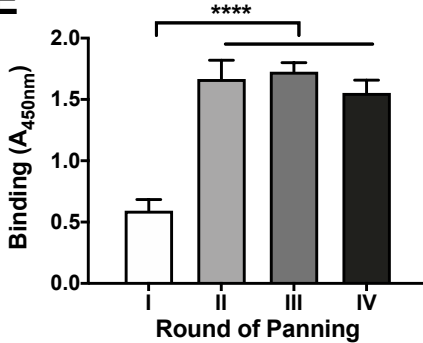
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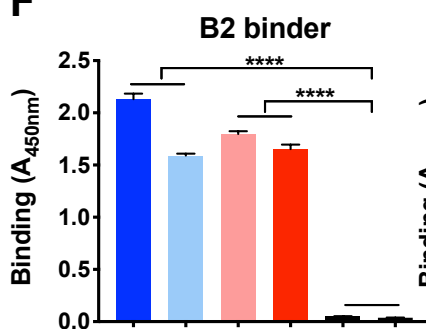
D



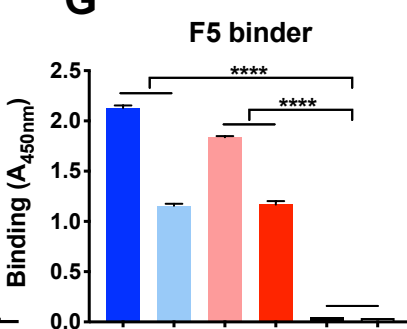
E



F



G



H

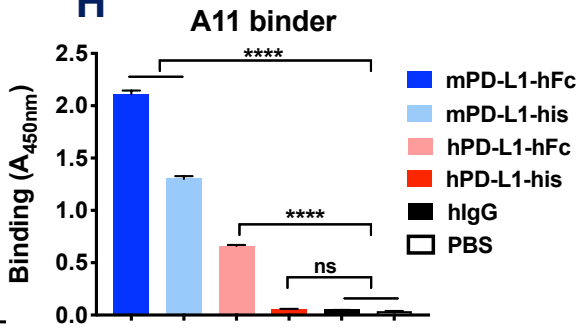


Figure 2

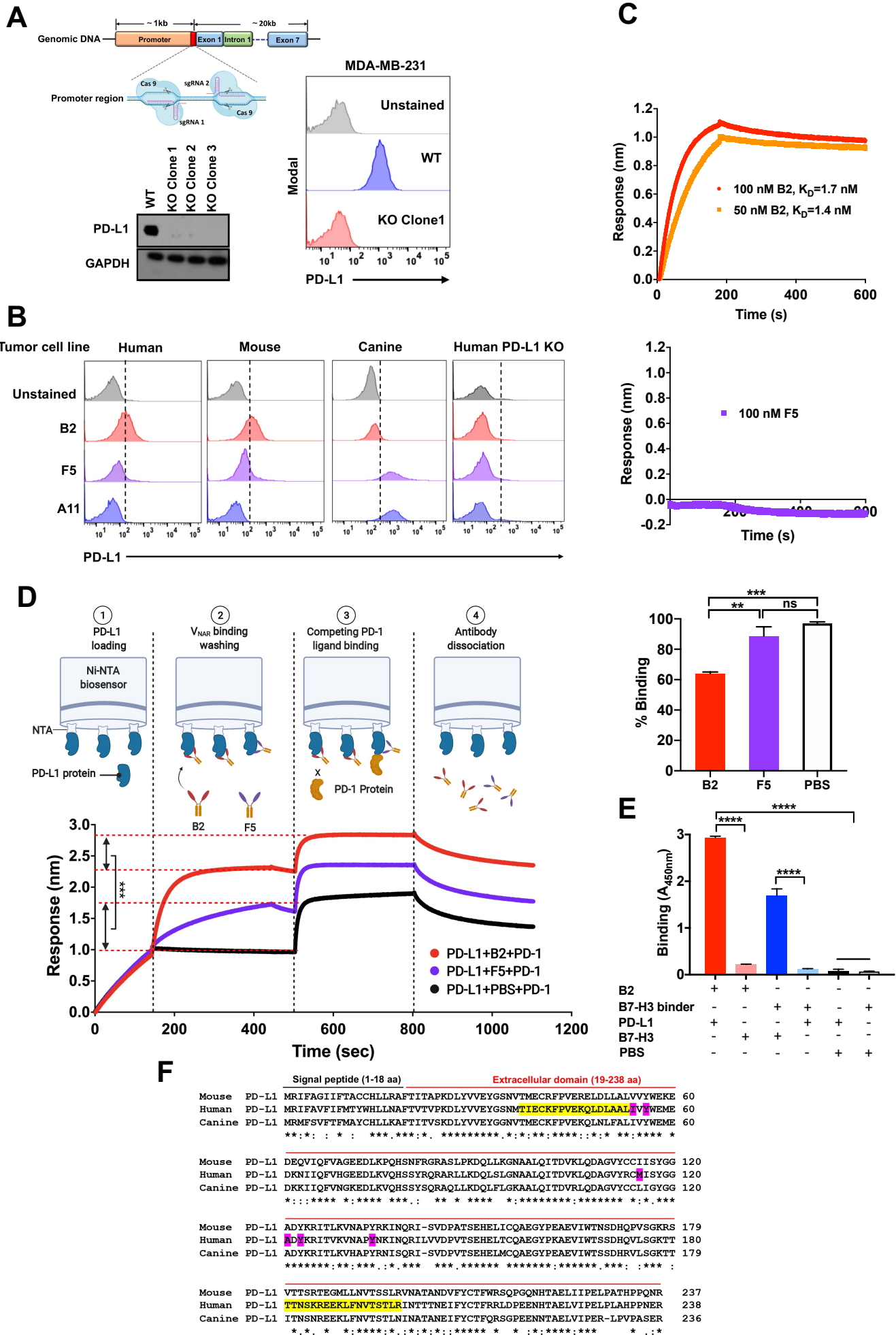
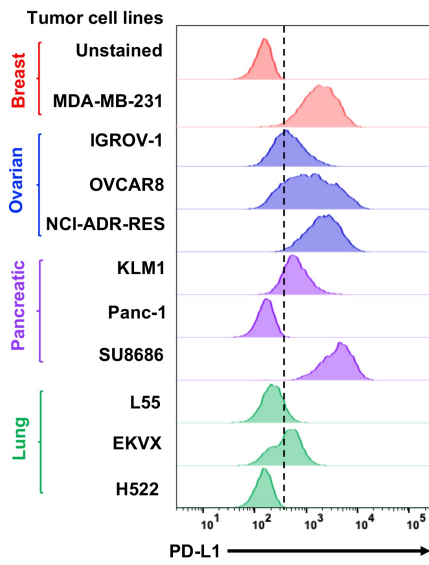
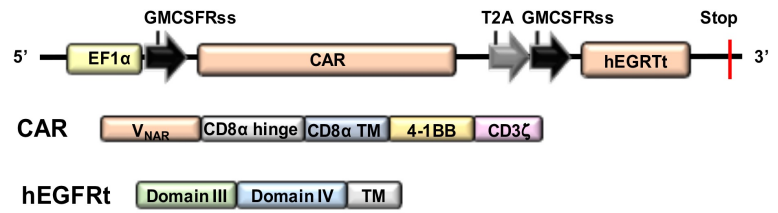


Figure 3

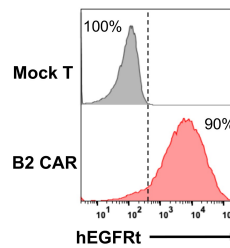
A



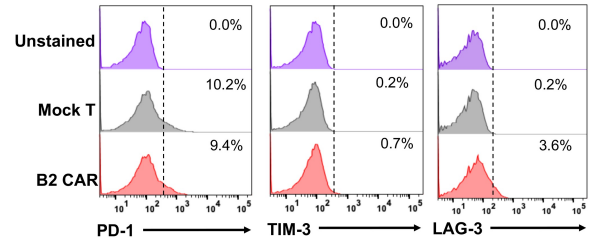
B



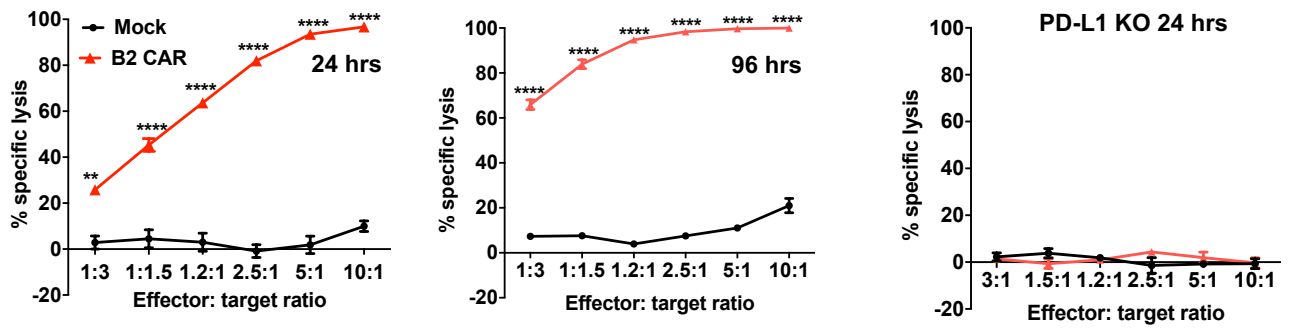
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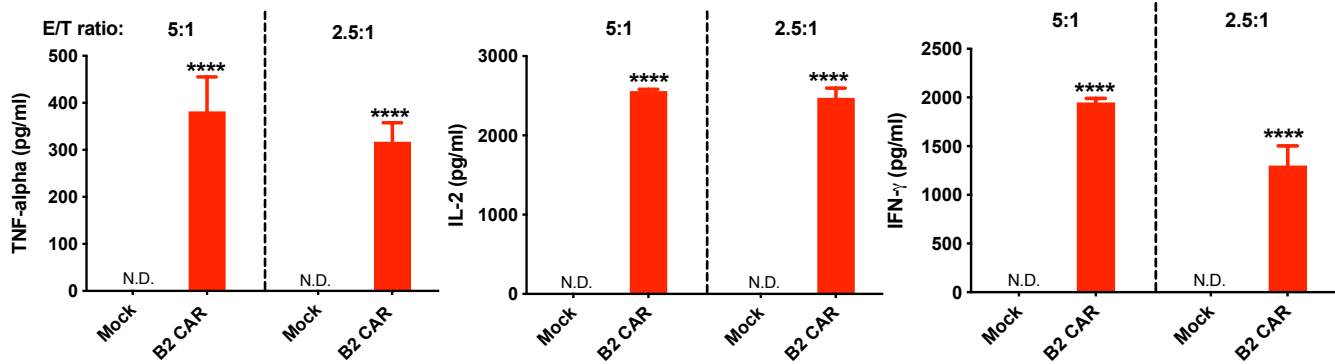
D



E



F



G

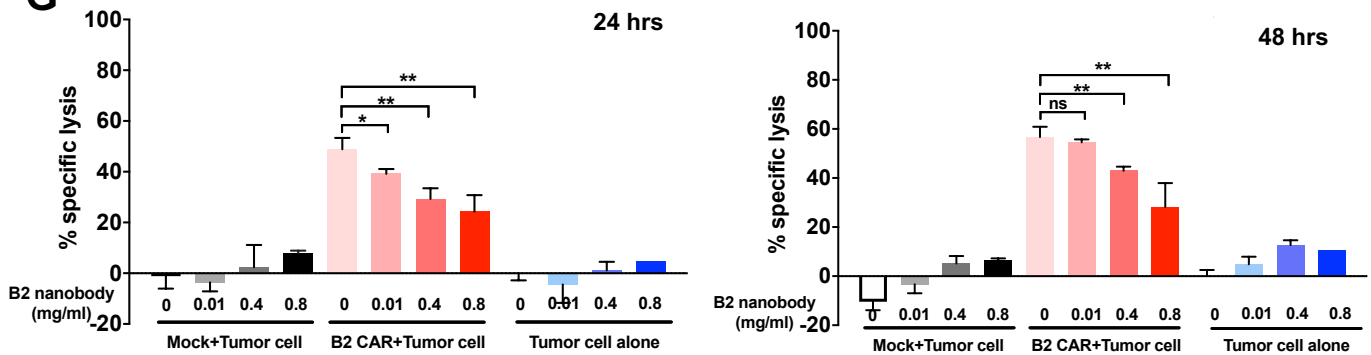
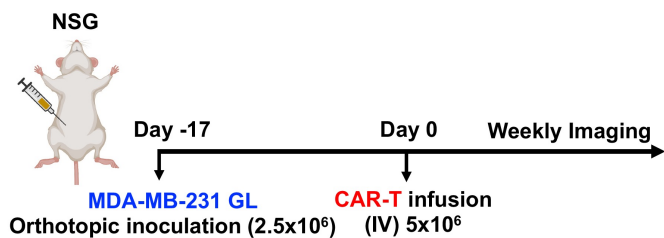
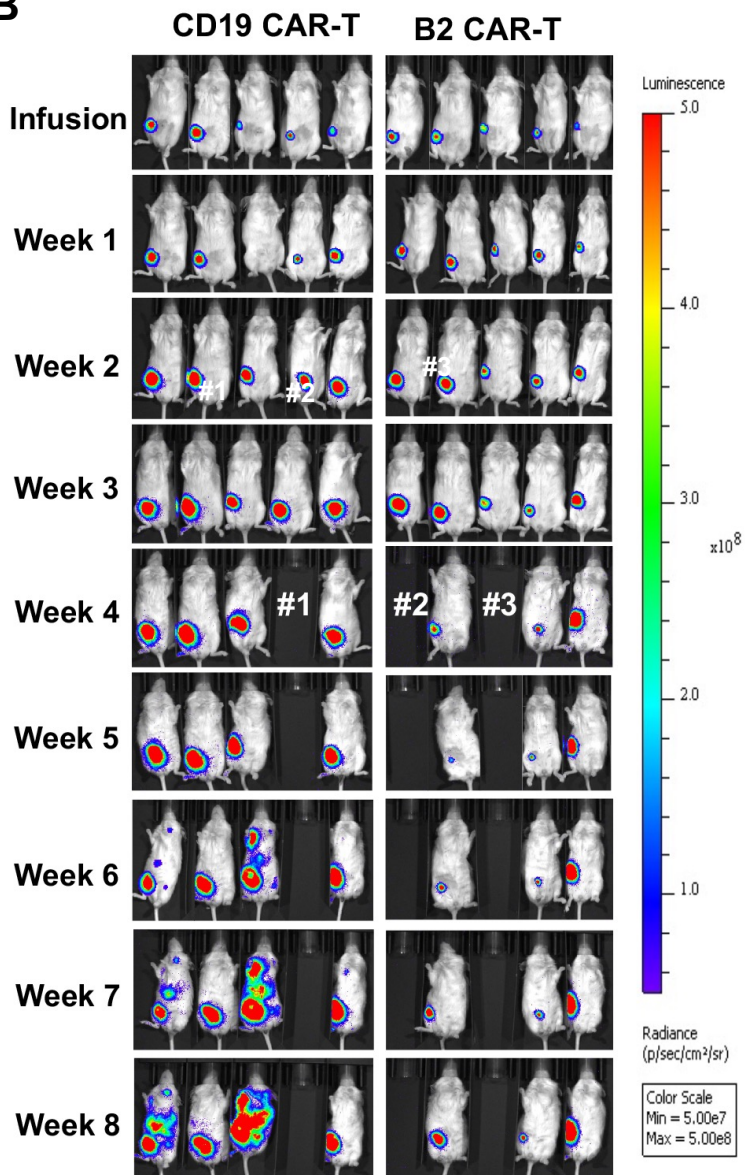


Figure 4

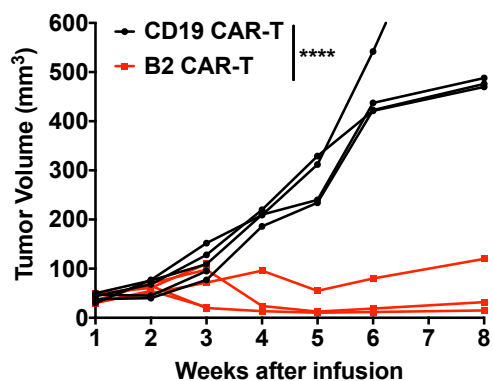
A



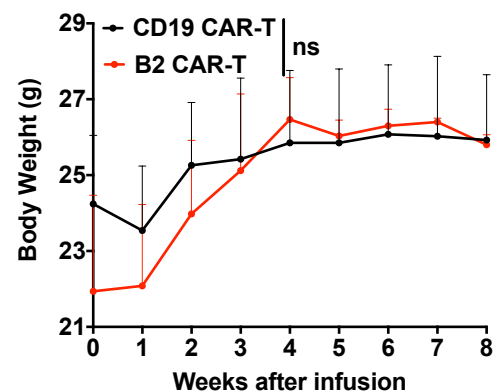
B



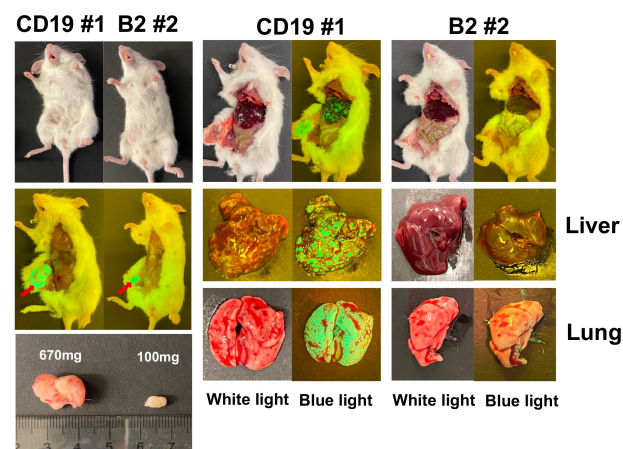
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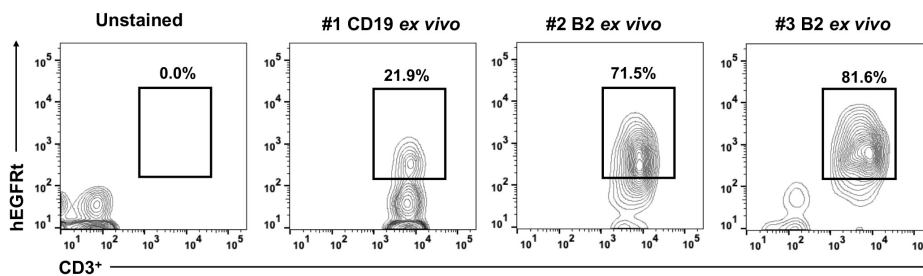
D



E



F



G

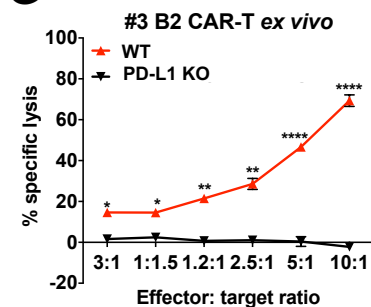


Figure 5

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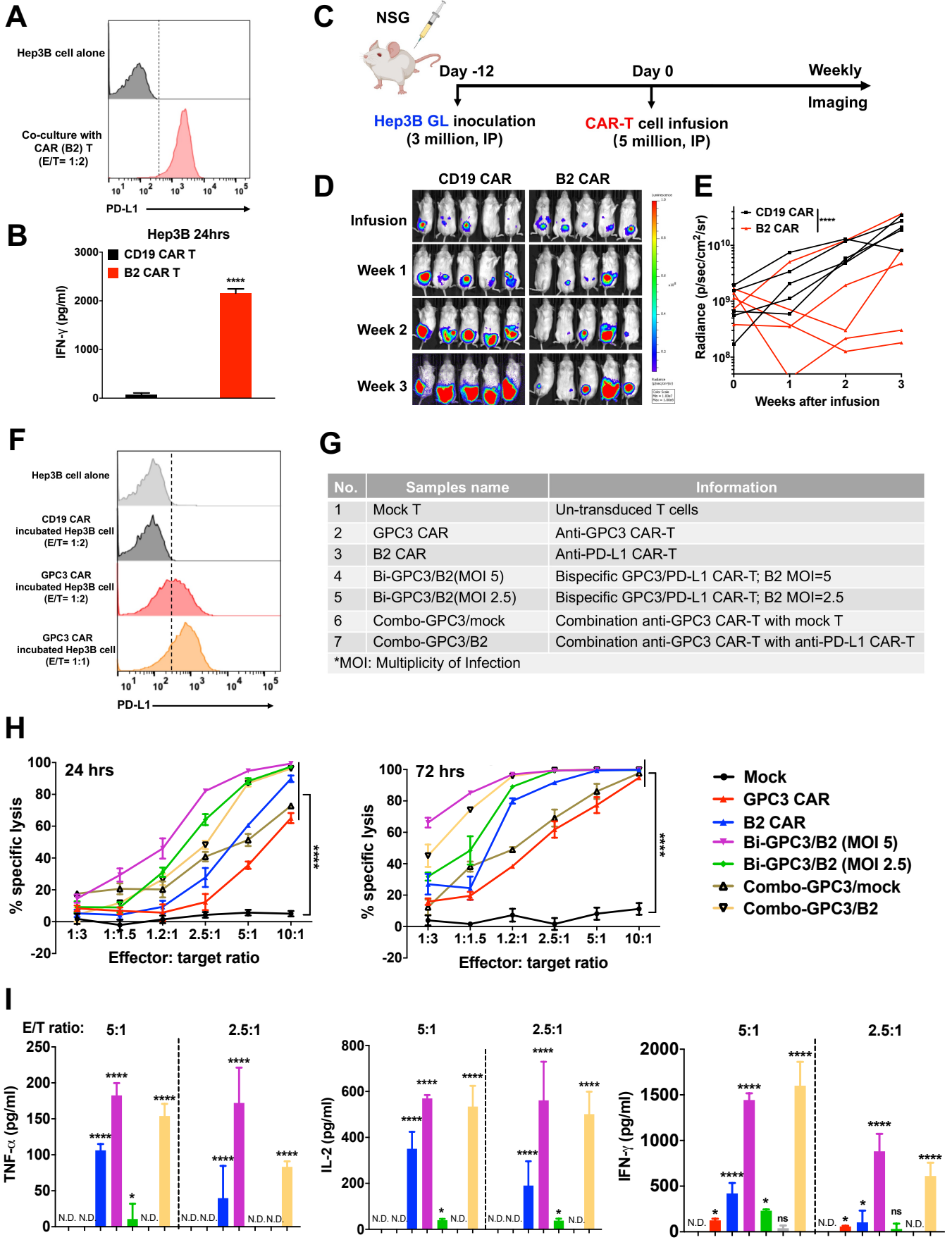


Figure 6

