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Supplemental Information

Chimeric Antigen Receptors Incorporating D Domains Targeting CD123 Direct Potent Mono- and Bi-specific Antitumor Activity of T Cells

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Supplemental Figures

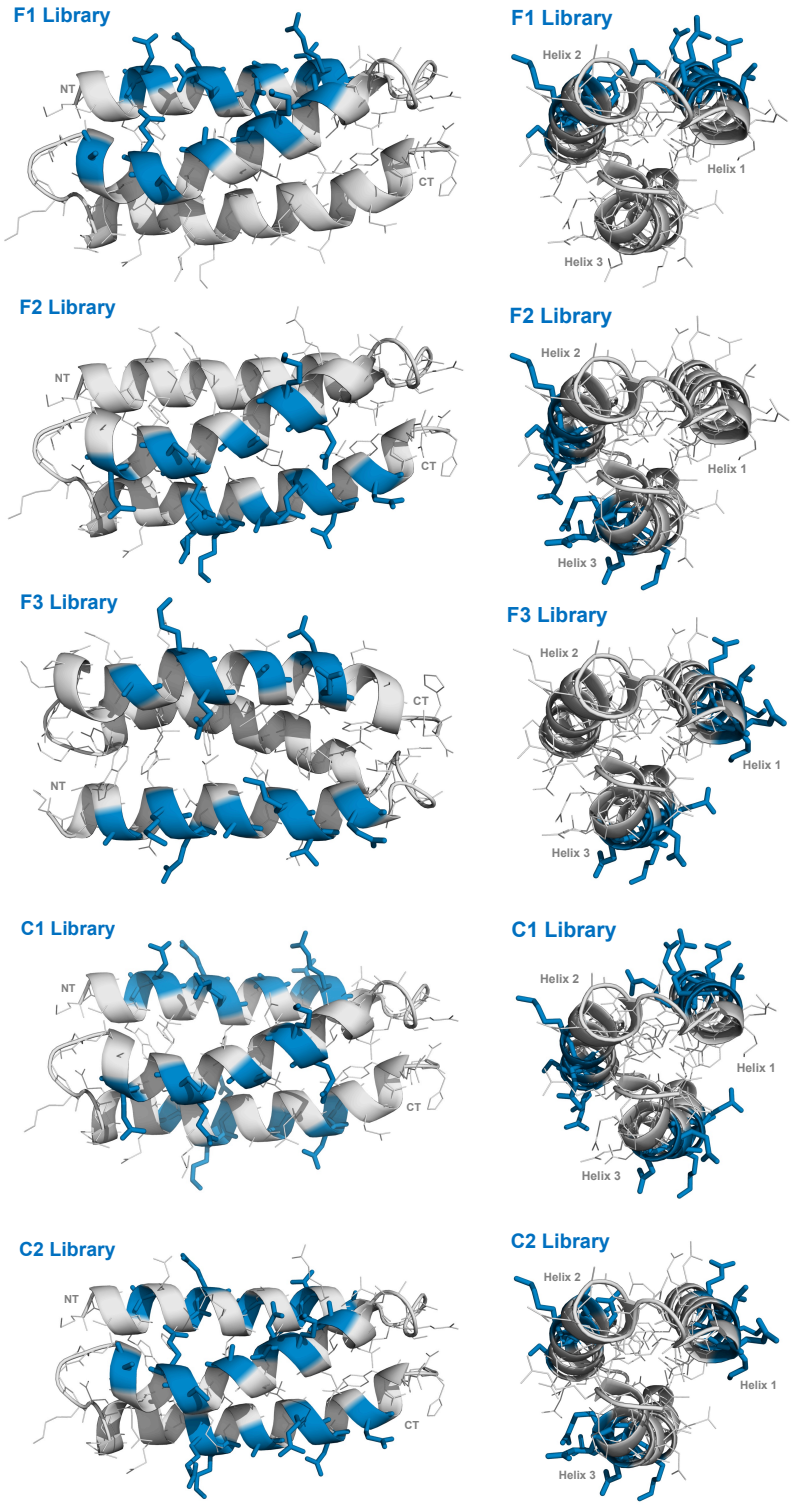

Figure S1. D domain library design. Libraries were designed using two architectures: Face libraries (F), in which mutated residues were selected from two adjacent helices and Combined libraries (C), in which residues from all three helices were used. F1 face is defined as helix 1 and helix 2. F2 face is defined as helix 2 and helix 3. F3 face is defined as helix 1 and helix 3. Models were created using PyMOL (v1.8) and based on the structure of α3D (PDB 2A3D). Two perspectives for each library design are shown, with randomized positions in blue and N-terminal (NT) and C-terminal (CT) residues are indicated.

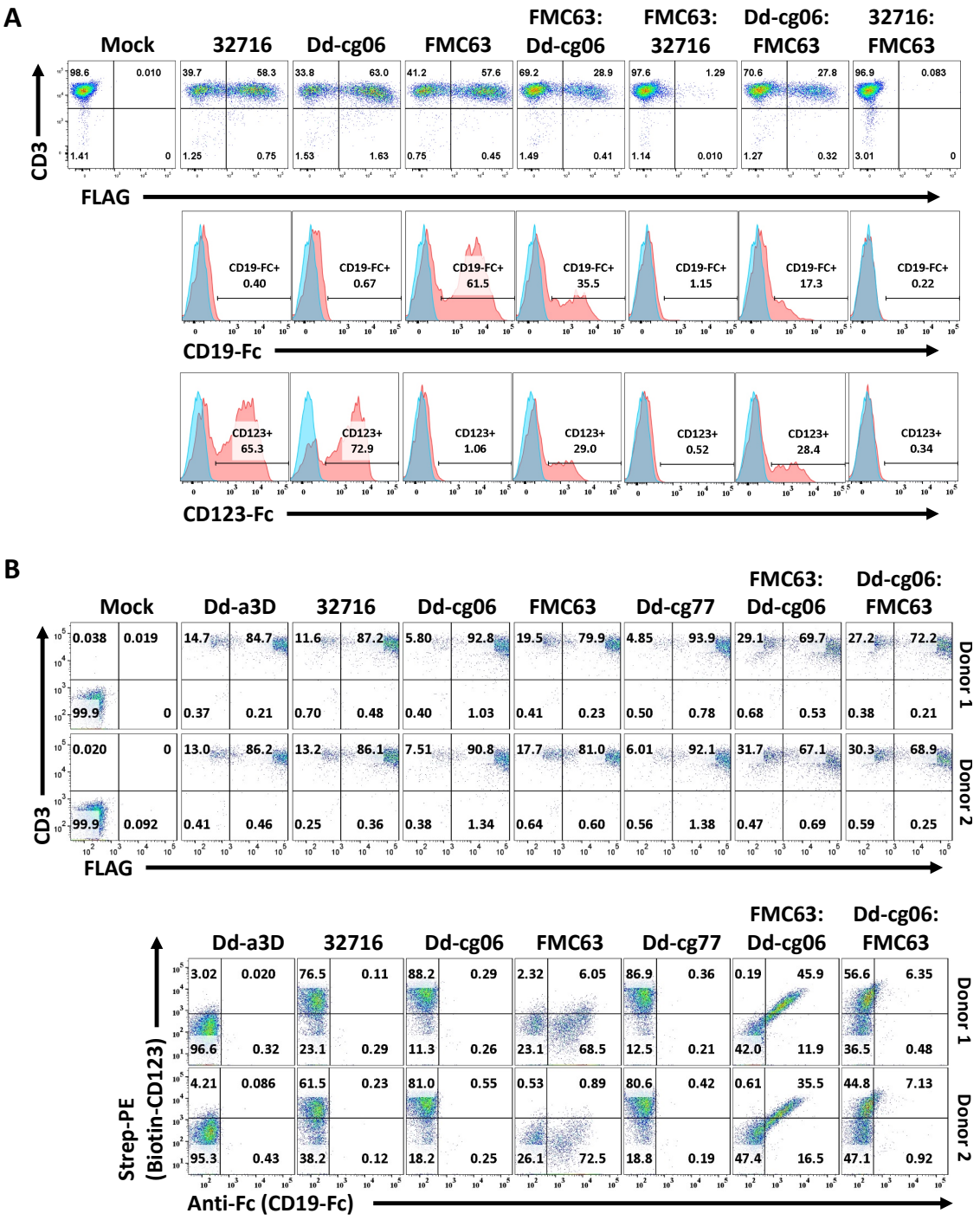

Figure S2: CAR expression and binding to soluble CD123 and CD19. Flow cytometry experiments were carried as described in **Methods and Materials**. **(A)** Analysis of CAR expression (CD3+ FLAG+) and binding of soluble target. For binding experiments, 10^5 T cells were washed and incubated with 0.5µg of hCD19-Fc or hCD123-Fc chimeric proteins (Sino and R&D Systems, respectively) for 20 minutes at 4°C in flow cytometry buffer, washed twice, then binding detected with PE anti-human Fc. **(B)** Analysis of CAR expression (CD3+ FLAG+) and simultaneous binding of soluble targets, for two donors. In binding experiments, T cells were co-incubated with hCD19-Fc and biotinylated-hCD123-His (R&D Systems; biotinylated in-house) simultaneously, washed twice, and binding detected with streptavidin-PE and A488 anti-Human Fc.

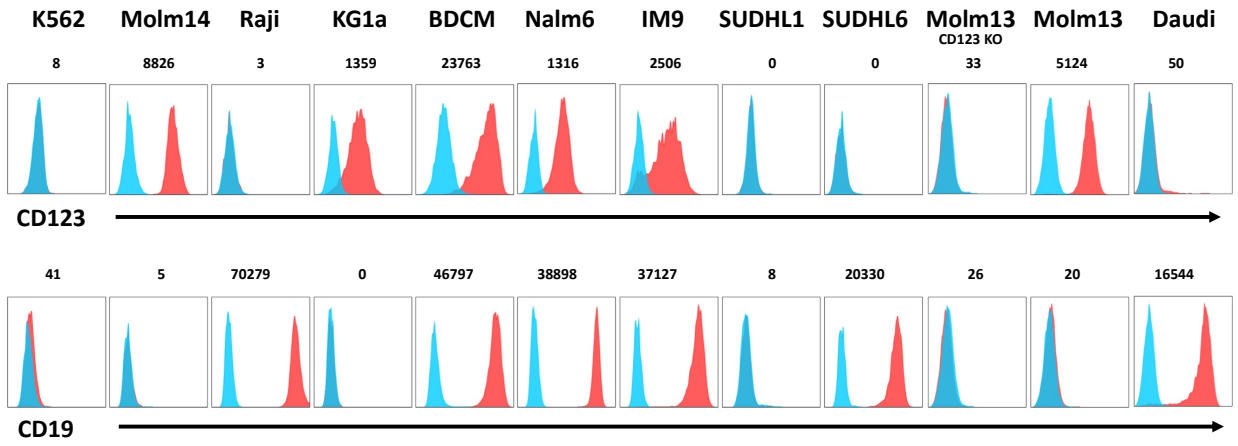

Figure S3: CD123 and CD19 expression on target cells. Expression histogram for cell lines used in this study. Delta MFI values, relative to isotype control (blue histogram) are indicated. Detection was performed using PE-anti human CD123 antibody (clone 6H6) and PE-anti human CD19 antibody (clone SJ25C1) from BioLegend.

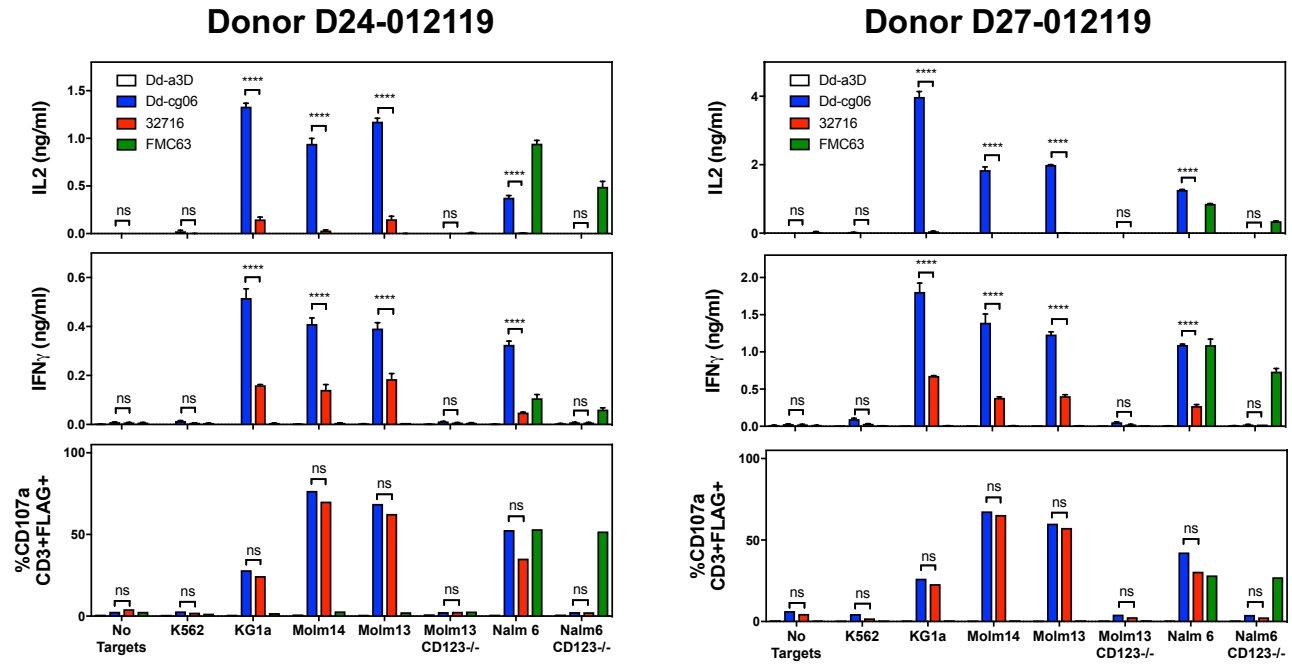

Figure S4: Cytokine secretion and degranulation of Dd-cg06 CAR T cells derived from two donors. Primary T cells from two donors, expressing Dd-cg06 CAR secrete IL2 and IFNγ (1 donor, n = 3, mean ± SEM) and degranulate (1 donor, n = 1) in response to target cells. K562 are CD123-/CD19-. KG1a are CD123+/CD19-. MOLM14 are CD123+/CD19-. MOLM13 are CD123+/CD19-. MOLM13 CD123-/- are CD123-/CD19-. NALM6 are CD123+/CD19+. NALM6 CD123-/- are CD123-/CD19+. CAR T cell response in the absence of target cells are also shown (No Targets). Cytokine and degranulation assessment was performed as described in **Methods and Materials**.

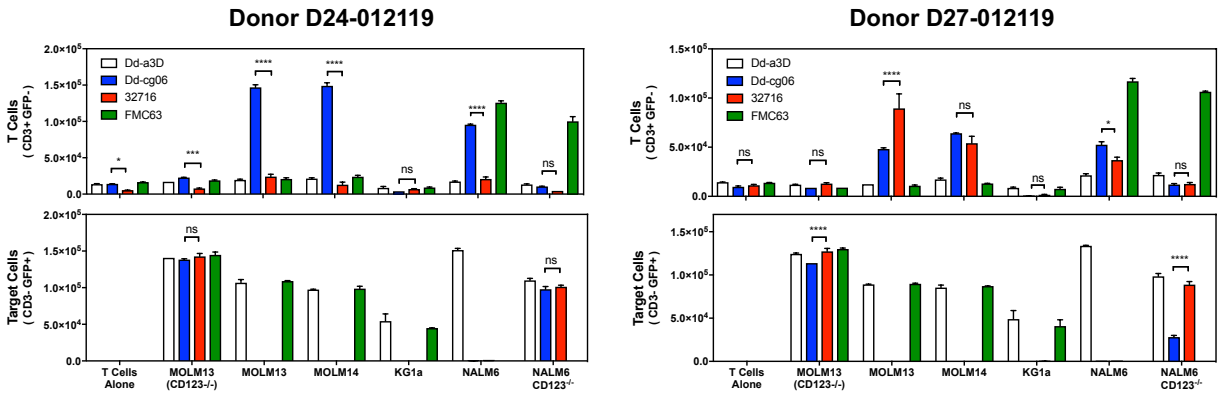

Figure S5: Target cell kill by and proliferation of Dd-cg06 CAR T cells derived from two donors. Nine days after transduction, cells were cultured overnight in media without IL2, then CFSE labeled and cultured for 4 days in the presence of untreated target cells (25,000 FLAG+ T-cells and 25,000 target cells). CD3+ CAR T cells and CD3- target cells are then counted by flow cytometry. In each panel, data represents mean ± SEM, triplicates of one donor. KG1a are CD123+/CD19-. MOLM14 are CD123+/CD19-. MOLM13 are CD123+/CD19-. MOLM13 CD123-/- are CD123-/CD19-. NALM6 are CD123+/CD19+. NALM6 CD123-/- are CD123-/CD19+. CAR T cell response in the absence of target cells are also shown (T cells Alone).

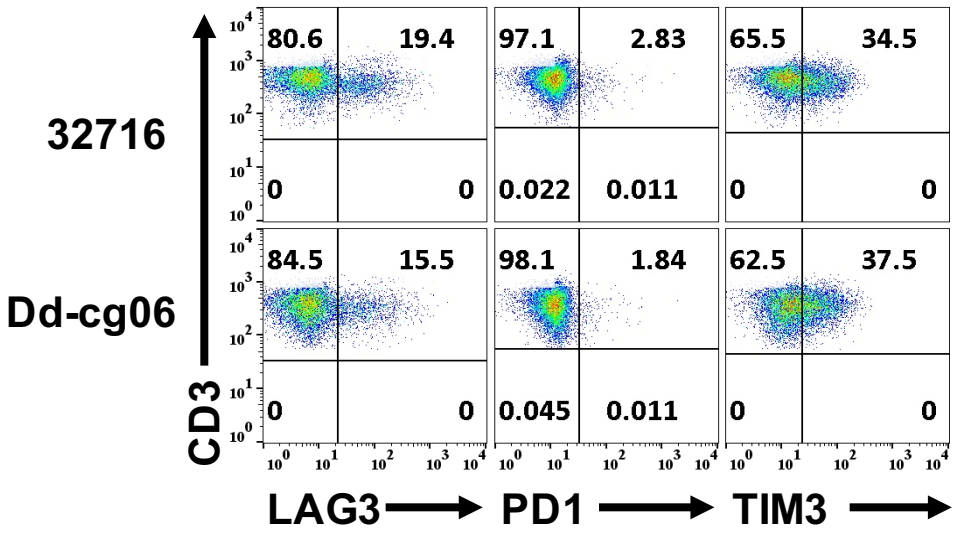

Figure S6: Representative FACS exhaustion data. Characterization of T cell exhaustion was performed as described in **Methods and Materials** and **Figure 3C**. Transduced T cells were stained with antibodies against CD3, LAG3, PD1, and TIM3, 9 days after activation with anti-CD3/anti-CD28 T cell activation beads in culture media supplemented with 40U/ml of IL2.

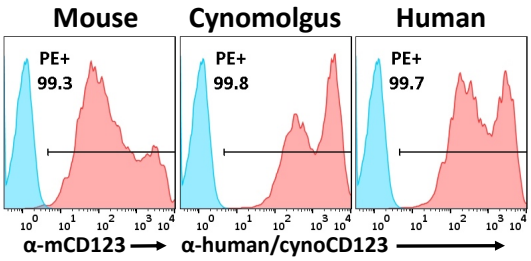

Figure S7: CD123 ortholog expression. DNA encoding human (NCBI Reference Sequence: NP_002174.1, residues 1 to 378), cynomolgus (UniProtKB sequence G8F3K3, residues 1 to 378) and murine (NCBI Reference Sequence: NP_032395.1, 1 to 396) CD123 was clone into pcDNA3 expression vector and transiently transfected into HEK 293 cells. Eighteen hours after transfection, cells were stained with antibody clone 5B11-PE (anti-mouse CD123) or antibody clone 7G3-PE (anti-human/cynomolgus monkey CD123). Percent positive values, relative to isotype control (blue histogram) are indicated.

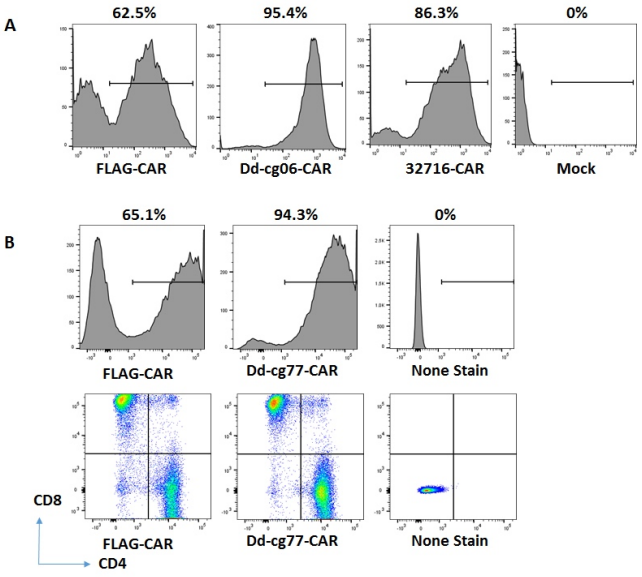

Figure S8A: CAR expression for transduced T cells used in *in vivo* studies. Experiments were carried as described in **Methods and Materials**. (**A**) CAR expression data for transduced T cells used in MOLM14 *in vivo* model from **Figure 3C**. (**B**) CAR expression data for transduced T cells used in KG1a *in vivo* model from **Figure 4C**.

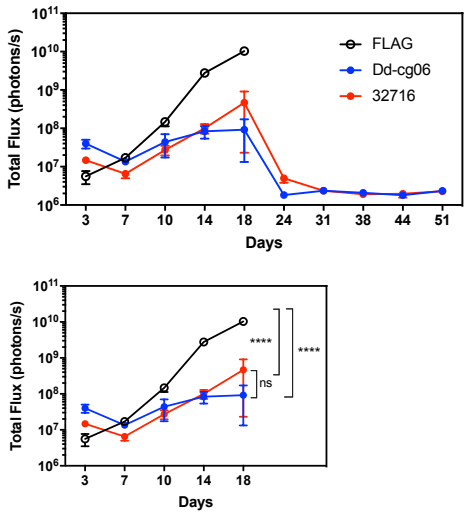

Figure S8B: Bioluminescent signal flux analysis for MOLM14 *in vivo* data from Figure 3C. Experiments were carried as described in **Methods and Materials**. Bioluminescent imaging using the Xenogen IVIS Lumina (Caliper Life Sciences). Mice were injected intraperitoneally with 3 mg D-luciferin (Caliper Life Sciences) and were imaged 4 minutes later with an exposure time 1 min. Living Image Version 4.3.1 SP2 software (Caliper Life Sciences) was used to analyze the bioluminescent signal flux (photons/s) for each mouse. Data were analyzed using GraphPad Prism 7 software (GraphPad). Values for 5 (Dd-cg06 and 32716) and 4 (FLAG) animal cohorts are represented as mean ± SEM for the entire course of the experiment (top panel). Two-way ANOVA statistical tests was performed through Day 18 (bottom panel) for grouped statistics, using Tukey correction for multiple comparisons. ns = not significant. **** $P < 0.0001$.

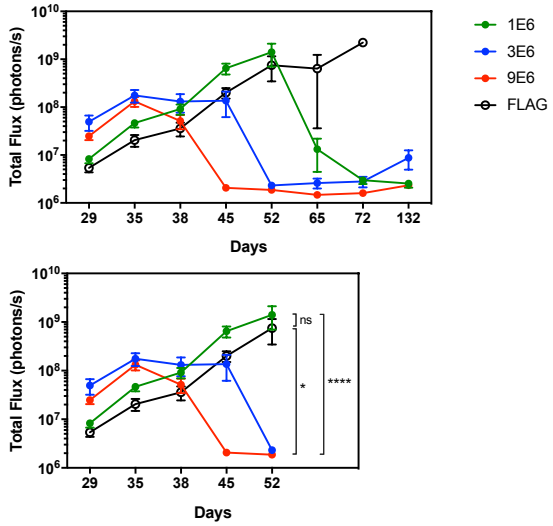

Figure S8C: Bioluminescent signal flux analysis for KG1a *in vivo* data from Figure 4C. Experiments were carried as described in **Methods and Materials**. Bioluminescent imaging using the Xenogen IVIS Lumina (Caliper Life Sciences). Mice were injected intraperitoneally with 3 mg D-luciferin (Caliper Life Sciences) and were imaged 4 minutes later with an exposure time 1 min. Living Image Version 4.3.1 SP2 software (Caliper Life Sciences) was used to analyze the bioluminescent signal flux (photons/s) for each mouse. Data were analyzed through using GraphPad Prism 7 software (GraphPad). Values for five animal cohorts are represented as mean ± SEM for the entire course of the experiment (top panel). Two-way ANOVA statistical tests was performed through Day 52 (bottom panel) for grouped statistics, using Tukey correction for multiple comparisons. ns = not significant. **** $P < 0.0001$.

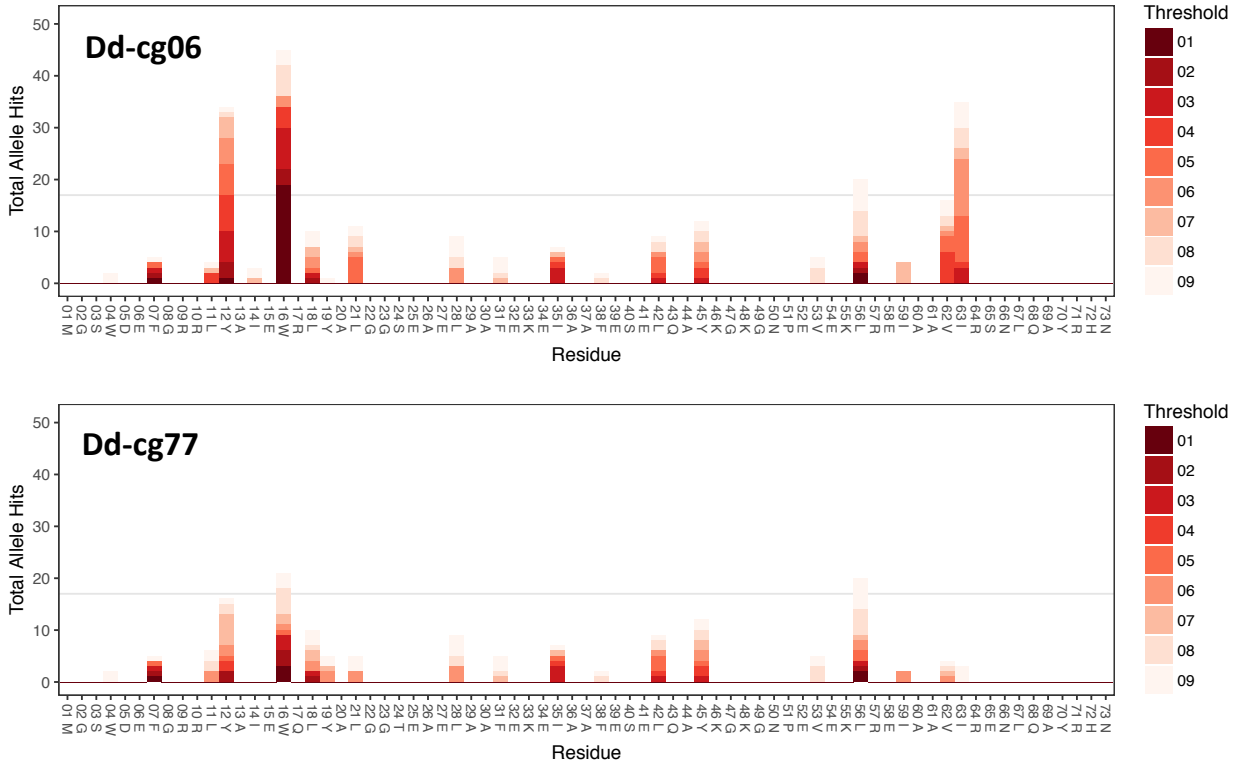

Figure S9: Deimmunization of Dd-cg06. D domain sequences were screened for T cell epitopes using a virtual matrix-based prediction algorithm for identification of Class II epitopes (Singh and Raghava, 2001). High affinity epitopes are defined as those present at 6% threshold and below. A total of 51 MHC HLA-DR alleles are screened. Promiscuous epitopes are defined as those with 17 (grey horizontal line) or greater hits. As defined, Dd-cg06 has three, high affinity, promiscuous epitopes, which are located at Y12, W16 & I63. Dd-cg77 was generated through the introduction of three mutations R17Q, S24T, S65E, which reduced the number of high affinity, promiscuous epitopes to zero.

Fc Fusion Protein	K_{on} ($M^{-1}s^{-1}$)	K_{off} (s^{-1})	K_D (M)	Ave K_D (M)
Dd-cg06-Fc	$4.61x10^{4}$	$6.53x10^{-4}$	$1.42x10^{-8}$	$1.44x10^{-8}$
	$4.43x10^{4}$	$6.46x10^{-4}$	$1.46x10^{-8}$	
Dd-cg77-Fc	$1.72x10^{5}$	$1.17x10^{-3}$	$6.79x10^{-9}$	$6.39x10^{-9}$
	$1.66x10^{5}$	$9.95x10^{-4}$	$5.99x10^{-9}$	
32716 scFv-Fc	$5.71x10^{5}$	$6.52x10^{-4}$	$1.14x10^{-9}$	$1.24x10^{-9}$
	$5.27x10^{5}$	$7.0x10^{-4}$	$1.33x10^{-9}$	
Dd-a3D-Fc	no binding	no binding	na	na

Table S1: Summary SPR data for binding of CD123-His to Fc fusion proteins. Summary of CD123 binding data described below in **Figure S10** and **Supplemental Methods and Materials**.

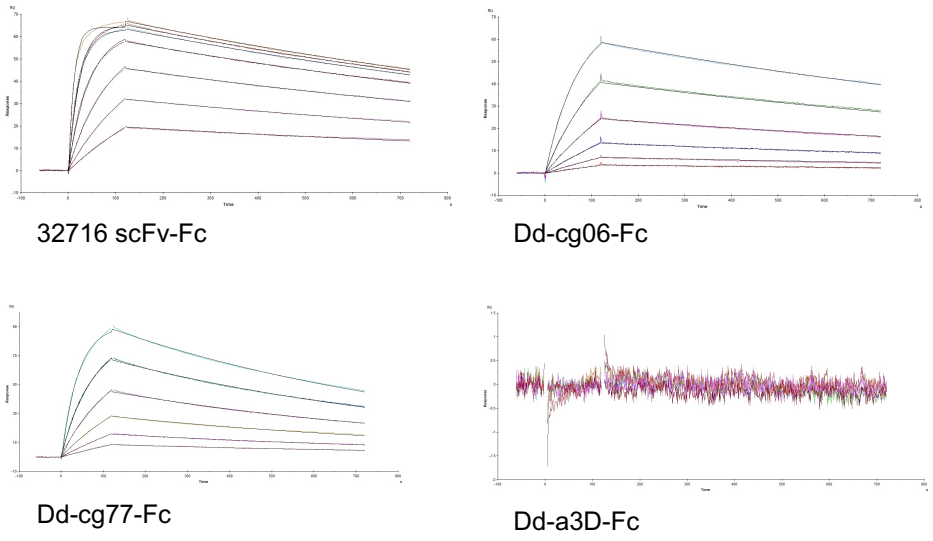

Figure S10: Representative sensorgrams for binding of CD123-His to Fc fusion proteins. Fc-fusions of Dd-cg06, Dd-cg77, Dd-a3D and 32716 scFv to human Fc were meditated via a 2xG_4S linker and were cloned into pcDNA3. Proteins were generated through transient transfection of CHO cells, followed by Protein A and SEC chromatography. Surface plasmon resonance experiments were performed using a Biacore X100 (GE Healthcare). For measuring binding affinity of Fc-fusion proteins to CD123-His, approximately 750 RU of Protein A was immobilized on Fc1 and Fc2 of a research-grade, CM4 sensor chip using standard EDC/NHS amine coupling chemistry. All experiments were performed in PBS, 3 mM EDTA, 0.05% Tween20, pH 8.5 as the binding buffer at 25°C. To collect binding kinetics data, approximately 100 RU of the Fc-fusion proteins were captured with a 60 sec injection of 10 nM protein. CD123-His was then injected at concentrations of 150, 75, 37.5, 18.75, 9.375 and 4.6875 nM at a flow rate of 30 µl/min. For Dd-cg06-Fc, 300 nM was used as the highest concentration and 9.375 nM as the lowest concentration. The surface was regenerated with a 60 sec injection of 10 mM Glycine pH 1.5. The data was fit to a 1:1 interaction model using the local Rmax fitting data analysis option available within the Biacore X100 Evaluation software.

MBP Fusion Protein	SPR			ELISA
	K_{on} ($M^{-1}s^{-1}$)	K_{off} (s^{-1})	K_D (M)	EC50 (M)
Dd-a3D-MBP	No binding	No binding	No binding	No binding
Dd-cg77-MBP	1.07E+05	7.75E-04	7.24E-09	9.89E-11
Dd-cg77(D5A)-MBP	1.08E+05	2.13E-03	1.97E-08	1.34E-10
Dd-cg77(G8K)-MBP	No binding	No binding	No binding	2.31E-08 *
Dd-cg77(R9Q)-MBP	1.02E+05	5.68E-04	5.57E-09	1.11E-10
Dd-cg77(Y12A)-MBP	No binding	No binding	No binding	1.89E-09
Dd-cg77(E15K)-MBP	No binding	No binding	No binding	2.86E-08 *
Dd-cg77(W16T)-MBP	6.82E+04	6.80E-03	9.98E-08	3.49E-10
Dd-cg77(Y19E)-MBP	No binding	No binding	No binding	4.81E-09
Dd-cg77(K55A)-MBP	1.44E+05	2.19E-03	1.52E-08	1.12E-10
Dd-cg77(E58K)-MBP	No binding	No binding	No binding	2.29E-08 *
Dd-cg77(I59E)-MBP	No binding	No binding	No binding	9.16E-10
Dd-cg77(V62A)-MBP	8.94E+04	1.83E-03	2.05E-08	1.14E-10
Dd-cg77(E65D)-MBP	9.53E+04	8.39E-04	8.81E-09	8.86E-11
Dd-cg77(N66E)-MBP	1.00E+05	3.24E-04	3.24E-09	7.81E-11

Table S2: Summary SPR and ELISA data for CD123-His to MBP fusion proteins. Summary of CD123 binding data described below in **Figure S11** and **S12**. * EC50 values for G8K, E15K and E58K mutants are not comparable, as ELISA binding curves are not parallel (**Figure S12**).

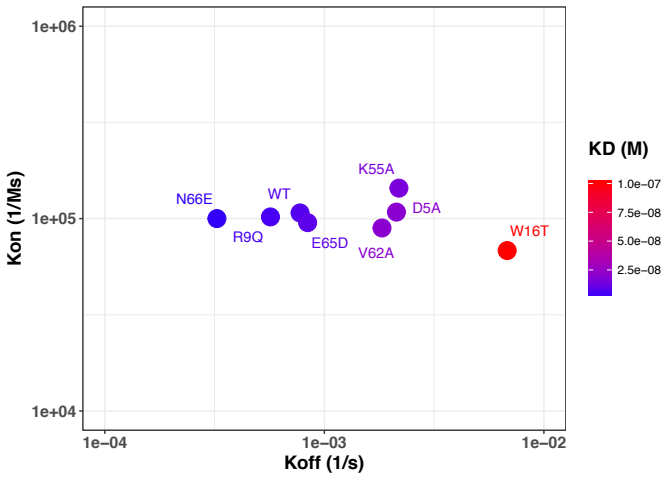

Figure S11A: Comparison of kinetic for data for CD123-His to MBP fusion proteins. Data is derived from sensorgrams described below in **Figure S11B**.

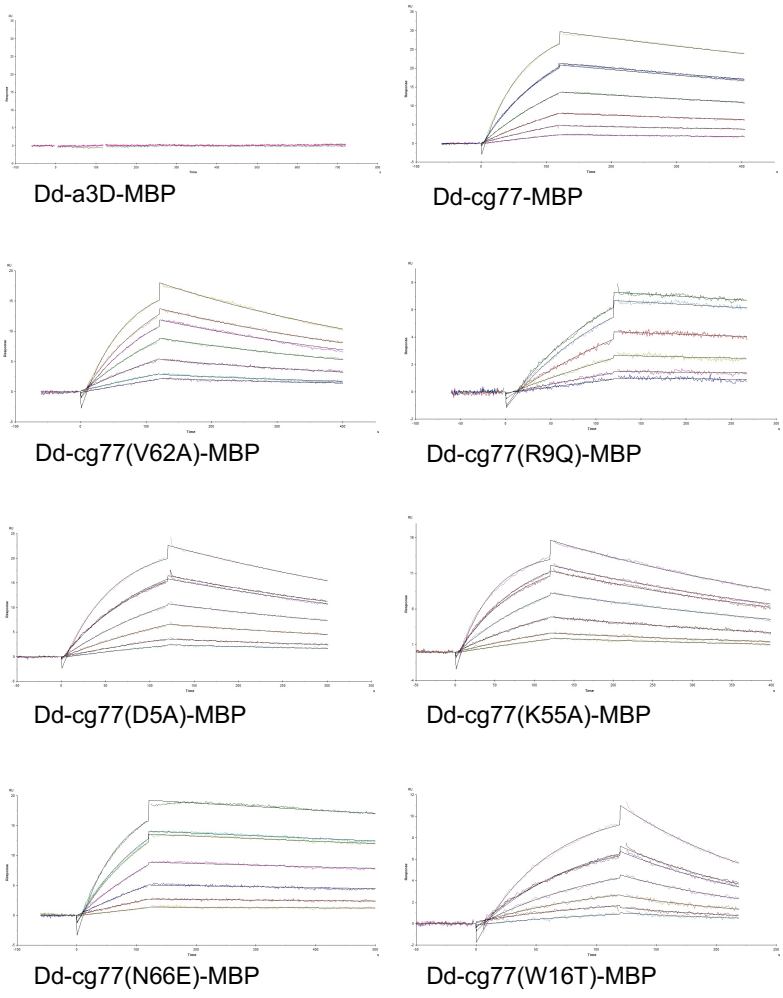

Figure S11B: Sensorgrams for the binding of CD123-His to MBP fusion proteins.

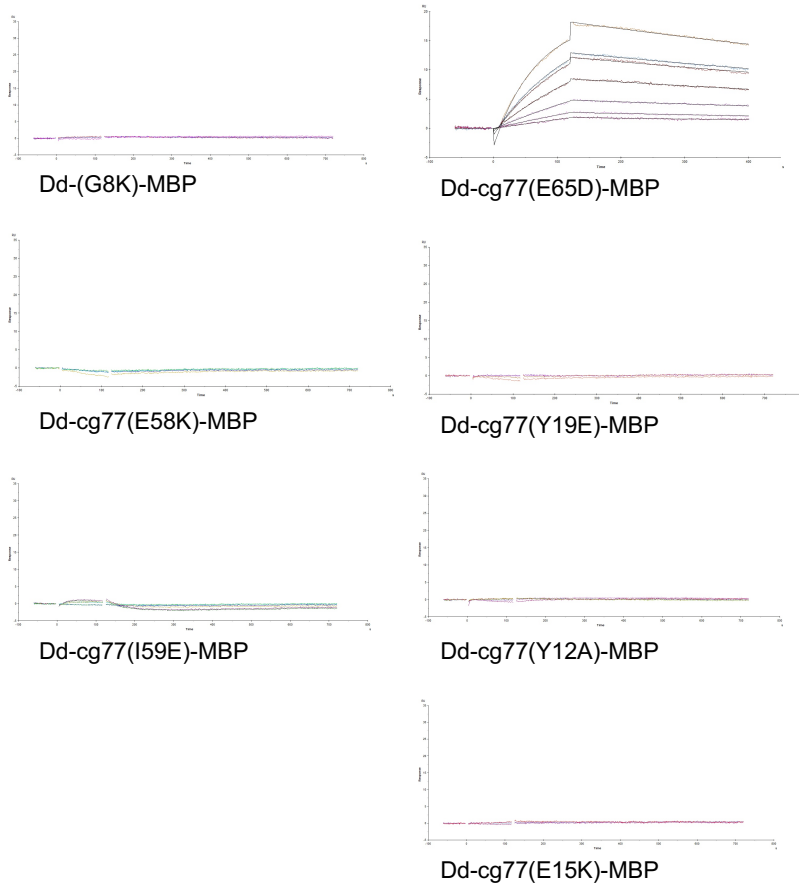

Figure S11B (Continued): Sensorgrams for the binding of CD123-His to MBP fusion proteins. Surface plasmon resonance experiments were performed using a Biacore X100 (GE Healthcare). For binding kinetics measurement of the various Dd-MBP fusion proteins to CD123-His, approximately 6000 RU of rabbit anti-MBP Polyclonal antibody (Abcam) was immobilized on Fc1 and Fc2 of a research-grade, CM4 sensor chip using standard EDC/NHS amine-coupling chemistry. Both surfaces were blocked with a 7 min injection of 1 M ethanolamine, pH 8.5 (GE Healthcare). All experiments were performed in PBS, 3 mM EDTA, 0.05% Tween20, pH 8.5 as the binding buffer at 25°C. To collect binding kinetics data, approximately 100 RU of MBP-fusion protein was captured with a 60-90 sec injection of 10 nM of the fusion protein. CD123-His (Sino Biological) was injected over the two flow cells at concentrations of 150, 75, 37.5, 18.75, 9.375 and 4.6875 nM at a flow rate of 30 µl/min. Binding response was measured during the association and dissociation times of 120 and 600 sec, respectively. Surfaces were regenerated with a 60 sec injection of 10 mM Glycine pH 2.0 and injections were done in a random order for each concentration. The data were fit to a simple 1:1 interaction model using the local Rmax fitting data analysis option available within Biacore X100 Evaluation software. For Dd-cg77 mutants that show no binding to CD123-His, the scale has been matched to the response obtained from CD123-His with Dd-cg77-MBP

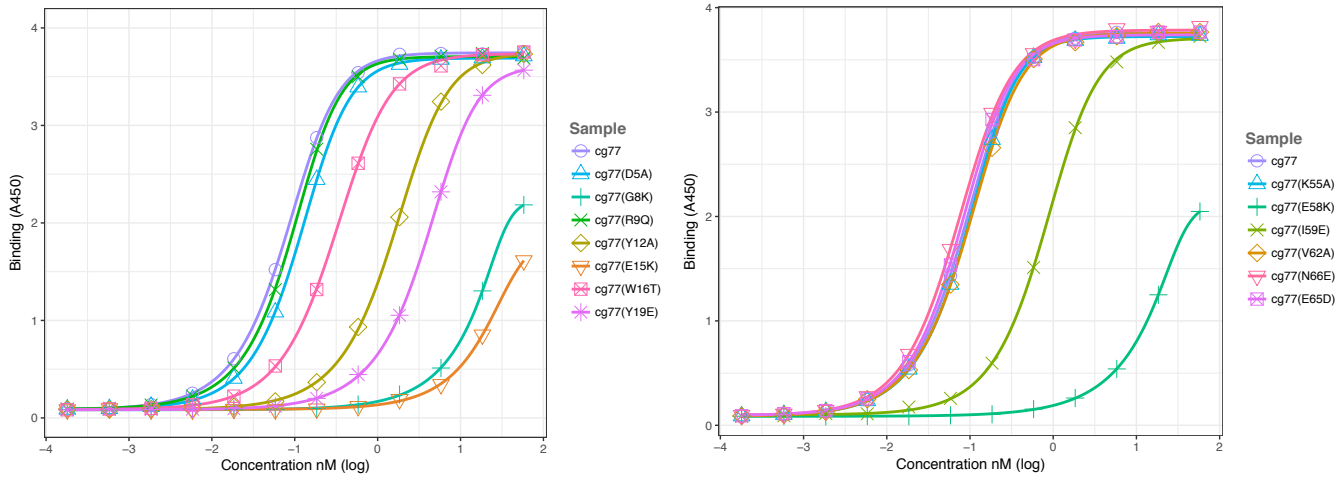

Figure S12: Binding of Dd-cg77 mutant MBP fusion proteins to CD123-Fc. MBP fusion proteins were generated, quantified and assayed in ELISA as described in **Methods and Materials**. EC50 values are listed in **Table S2**.