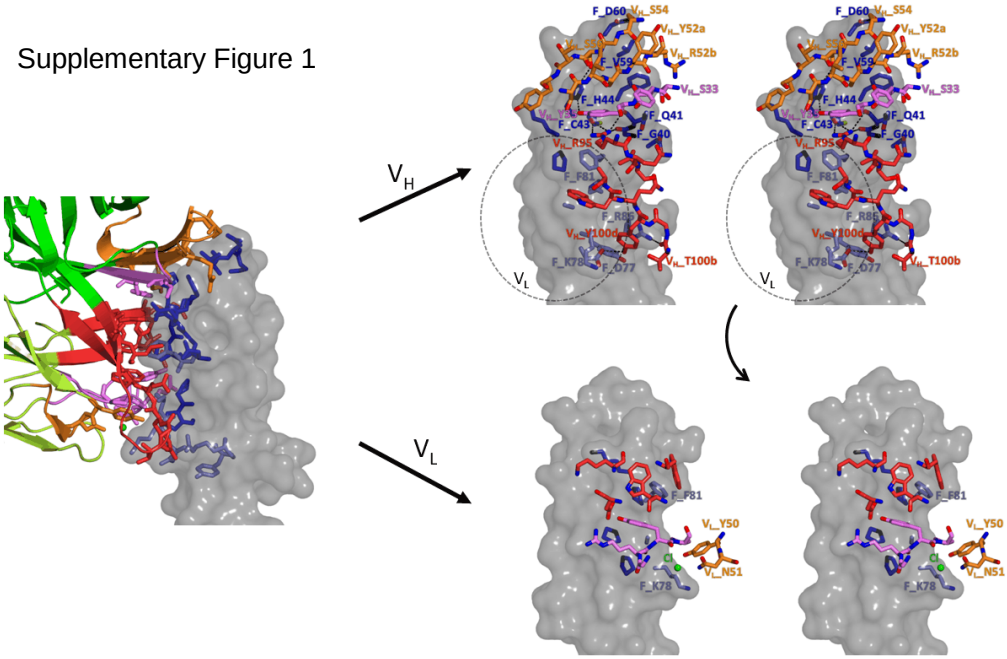
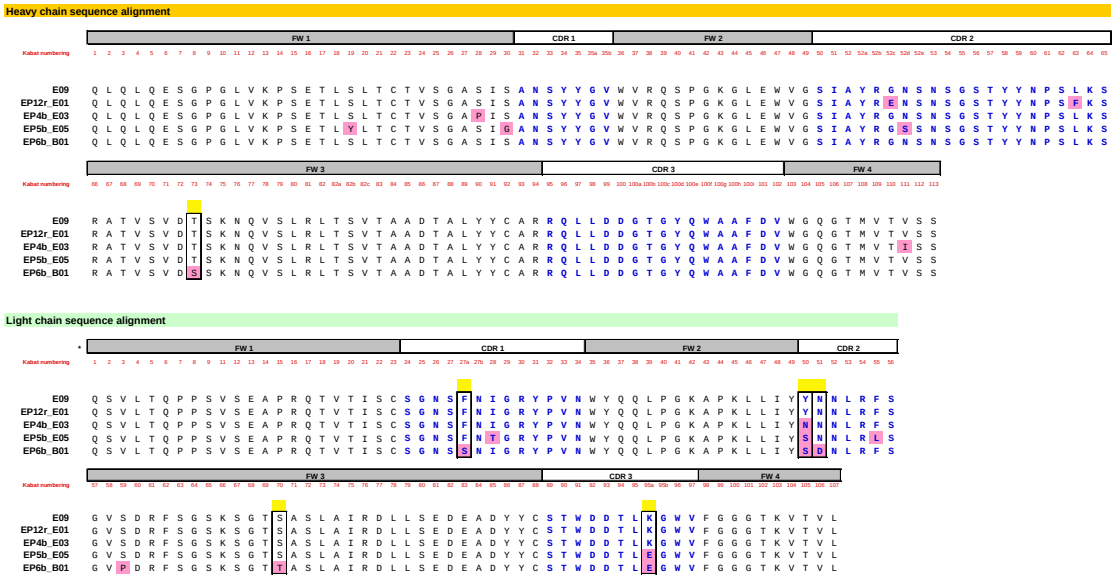
Supplementary Figure 1



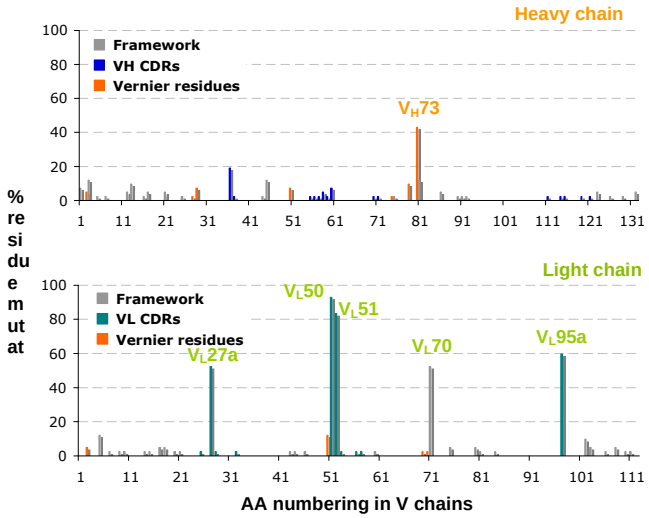
Supplementary Figure 1: Interface of the E09:Fas structure. The two interfaces formed by V_H and V_L of E09 with Fas are shown in stereo. The Fas receptor is represented as a surface with the epitope residues displayed as blue and light blue sticks for CRD1 and CRD2, respectively. The paratope residues of CDRs 1, 2 and 3 of V_H and V_L are represented as sticks and colored violet, orange and red, respectively. In the V_H epitope the position of the V_L epitope is indicated by the dashed circle.

Supplementary Figure 2



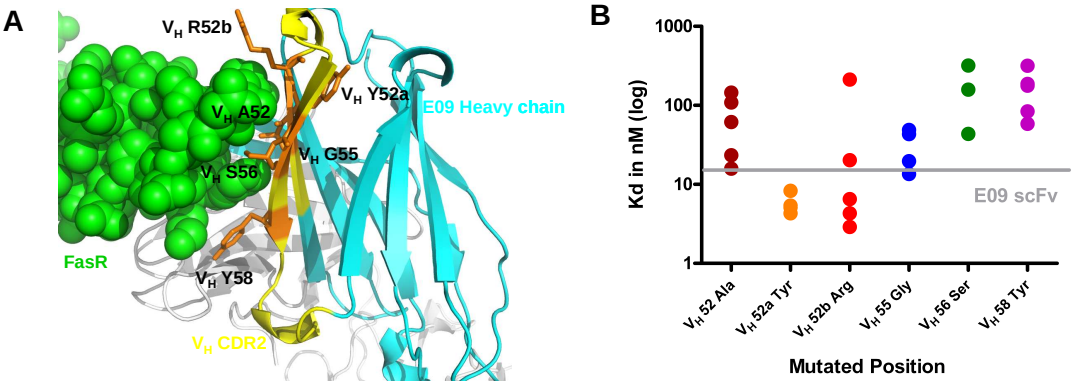
Supplementary Figure 2: Amino acid sequence alignment for E09 and variant antibodies. Heavy (top) and light (bottom) variable domain sequences are aligned versus the parental E09 antibody and differences are shown in pink. Hotspot mutations found in the round 6 population are highlighted in boxes.

Supplementary Figure 3



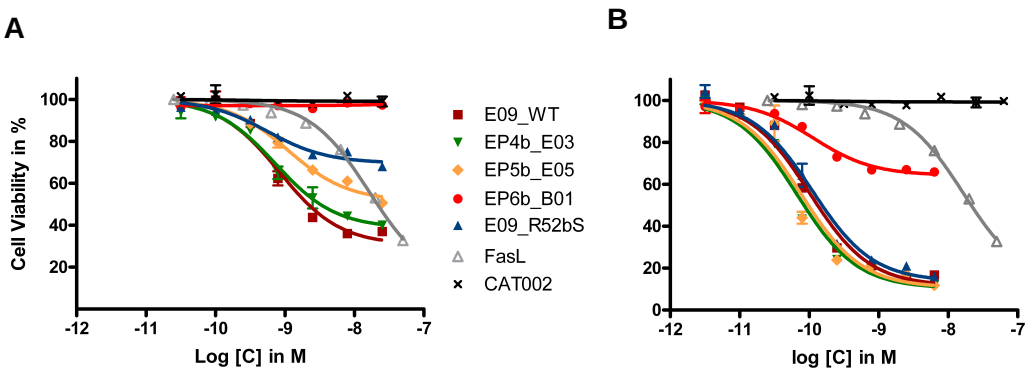
Supplementary Figure 3: Analysis of mutations after the directed evolution process. The percentage of residues mutated following 6 rounds of directed evolution selection is shown for both the heavy (top) and light (bottom) chains. Amino acids are numbered numerically. Positions mutated above 20% were defined as hotspots. Residues in the framework regions, the CDR loops and at Vernier positions are shown in grey, blue and orange bars, respectively.

Supplementary Figure 4



Supplementary Figure 4: Fas receptor affinity modulation by single point mutagenesis. A) Close-up view of the E09 heavy chain (cyan) in complex with the cysteine-rich repeat domain (CRD)-1 of Fas (green). Heavy chain CDR2 loop is shown in yellow and the 6 residues chosen for site-directed mutagenesis are shown in orange. B) Affinity of single point-mutants for Fas receptor as determined by SPR. Point mutants were purified as scFv and run as analyte on Fas ECD in fusion with Fc fragment previously immobilised on Protein G-immobilised CM5 chip. Points, each one representing a different sidechain replacement, were grouped by the residue mutated and sorted by K_d value on the Y-axis. Affinity of the parent E09 is shown as a grey line.

Supplementary Figure 5



Supplementary Figure 5: Influence of cross-linking IgGs on cell viability. Dose-dependent *in vitro* cell killing of Jurkat cells by anti-Fas E09 and variant antibodies without (A) or with (B) protein A cross-linking prior to the assay.

Supplementary Table 1

A. Diffraction data	sFasR:E09	EP6b_B01:sFasR
Space group	C2221	P3121
Unit cell constants [Å]/ [°]	a= 89.49, b= 166.40, c= 110.72; α=β=γ=90	a=b= 94.51, c= 139.20 α=β= 90, γ=120
Resolution [Å]	46.09 – 1.93 (1.98-1.93) [a]	47.26-2.10 (2.15-2.10)
Rsym [%]	4.5 (43.9)	10.4 (63.7)
I/σ	22.51 (4.73)	16.04 (4.13)
No. of reflections	398919	425950
No. of unique reflections	61296	42586
Completeness [%]	98.3 (99.6)	100 (100)
A. Refinement		
No. of reflections work / test	58229 / 3064	40441 / 2129
Rwork/Rfree	0.200/0.225	0.182 / 0.224
Number of atoms	8568 (incl. H)	8204 (incl. H)
Number of residues		519
Number of heteromolecules	2 Cd, 1 Cl, 6 EDO	2 NAG, 1 FUC, 5 EDO, 1 Na
Number of waters	411	349
Rmsd (bonds) [Å]	0.008	0.006
Rmsd (angles) [°]	1.104	0.955
DPI		
Biso [Å2]	49.06	39.85
Wilson B [Å2]	34.83	29.48
Ramachandran plot (Molprobity)		
Residues in most favored region [%]	95.9	96.7
Outliers [%]	0.2	0
Molprobity score	1.32	1.35

[a] Numbers in parentheses refer to the highest resolution shell

Supplementary Table 2

Fas	Residue	ASA	BSA	%	ΔiG	additional Interaction
39	Asp	113.34	26.71	23.6	-0.14	
40	Gly	16.42	8.16	49.7	0.01	H-bond
41	Gln	158.17	115.93	73.3	-0.91	H-bond
42	Phe	110.85	90.97	82.1	1.37	
43	Cys	13.63	12.8	93.9	-0.14	H-bond
44	His	159.67	149.84	93.8	0.75	H-bond
45	Lys	132.81	66.64	50.2	0.45	
46	Pro	70.77	65.56	92.6	0.92	
47	Cys	0.47	0.47	100.0	0.01	
48	Pro	64.61	47.06	72.8	0.75	
49	Pro	60.24	22.59	37.5	0.36	
58	Thr	80.89	3.19	3.9	-0.04	
59	Val	109.77	93.45	85.1	0.97	
60	Asn	136.96	12.25	8.9	-0.2	
61	Gly	42.6	0.74	1.7	-0.01	
75	Tyr	18.64	3.19	17.1	-0.04	
76	Thr	5.37	0.34	6.3	0.01	
77	Asp	66.7	14.77	22.1	-0.16	H-bond
78	Lys	146.82	64.18	43.7	0.64	
79	Ala	57.03	54.31	95.2	0.34	
80	His	35.43	17.32	48.9	0.28	
81	Phe	90.24	85.64	94.9	1.33	Cation-Pi
86	Arg	150.11	73.57	49.0	-1.37	H-bond / Cation-Pi

Supplementary Table 2: Interface residues in the E09:Fas complex determined by PISA. The antibody residues are numbered with the Kabat system. The accessible surface area (ASA) and the buried surface area (BSA) in Å2 of the indicated interface residues are indicated with the change in percent upon complex formation. The solvation energy effect is shown as ΔiG in kcal/mol. Interaction types are also listed.

Supplementary Table 2 (continued)

E09 V_L	Residue	ASA	BSA	%	ΔiG	additional Interaction
29	Gly	35.57	0.74	2.1	-0.01	
30	Arg	132.17	48.59	36.8	0.09	
31	Tyr	48.59	48.22	99.2	0.22	
32	Pro	19.4	13.32	68.7	0.21	
50	Tyr	41.42	31.13	75.2	0.05	
51	Asn	27.62	10.33	37.4	-0.12	
91	Trp	56.48	53.55	94.8	0.74	
93	Asp	41.34	3.81	9.2	-0.05	
95A	Lys	124.46	13.85	11.1	0.21	
96	Trp	16.98	8.34	49.1	0.09	
E09 V_H	**Residue**	**ASA**	**BSA**	**%**	**ΔiG**	**additional Interaction**
33	Ser	67.8	41.47	61.2	0.06	
34	Tyr	17.15	3.24	18.9	0.05	
35	Tyr	42.96	40.63	94.6	0.22	H-bond
50	Ser	1.46	1.34	91.8	0.02	
51	Ile	7.87	0.17	2.2	0	
52	Ala	41.35	40.59	98.2	0.47	
52A	Tyr	5.69	2.74	48.2	0.02	
52B	Arg	126.28	40.23	31.9	-0.09	
52E	Ser	65.91	7.54	11.4	0.02	
54	Ser	61.25	20.8	34.0	-0.05	
55	Gly	37.95	8.9	23.5	0.12	
56	Ser	75.16	47.26	62.9	0.13	H-bond
57	Thr	57.92	4.96	8.6	0	
58	Tyr	85.96	48.04	55.9	0.65	
95	Arg	58.15	57.53	98.9	-2.97	H-bond / Cation-Pi
96	Gln	64.18	7.25	11.3	-0.11	
97	Leu	100	70.21	70.2	1.05	
100B	Thr	95.72	9.82	10.3	-0.11	H-bond
100C	Gly	66.05	11.36	17.2	0.08	
100D	Tyr	134.1	98.37	73.4	0.6	H-bond/Cation-Pi
100E	Gln	100.78	36.39	36.1	-0.42	
100F	Trp	46.55	45.97	98.8	0.74	
100H	Ala	5.81	5.53	95.2	0.09	

Supplementary Table 3

E09 mutants (scFv)

#	scFv	Mutation	kd (1/s)	ka (1/Ms)	KD (M)	Change KD/parent
1	E09_WT	/	2.4E-03	2.8E+05	8.6E-09	1.0
2	E09_H73S	H73_T>S	1.3E-03	3.6E+05	3.5E-09	2.4
3	E09_L27aS	L27a_F>S	2.0E-03	2.5E+05	7.9E-09	1.1
4	E09_L50S	L50_Y>S	2.9E-04	3.3E+05	8.7E-10	9.8
5	E09_L51D	L51_N>D	5.0E-04	4.5E+05	1.1E-09	7.8
6	E09_L59P	L59_S>P	2.6E-03	3.3E+05	8.0E-09	1.1
7	E09_L70T	L70_S>T	2.6E-03	3.0E+05	8.5E-09	1.0
8	E09_L95aE	L95aE_K>E	1.9E-03	2.9E+05	6.7E-09	1.3

EP6bB01 revertants (scFv)

#	scFv	Mutation	kd (1/s)	ka (1/Ms)	KD (M)	Change KD/parent
1	EP6B_WT	/	7.8E-05	4.2E+05	1.8E-10	1.0
2	EP6B_H73S	H73_S>T	9.3E-05	4.8E+05	2.0E-10	1.1
3	EP6B_L27aS	L27a_S>F	1.0E-04	5.6E+05	1.8E-10	1.0
4	EP6B_L50S	L50_S>Y	1.4E-04	3.9E+05	3.7E-10	2.0
5	EP6B_L51D	L51_D>N	1.1E-04	3.2E+05	3.3E-10	1.8
6	EP6B_L59P	L59_P>S	1.3E-04	4.1E+05	3.1E-10	1.7
7	EP6B_L70T	L70_T>S	8.4E-05	5.2E+05	1.6E-10	0.9
8	EP6B_L95aE	L95aE_E>K	1.0E-04	5.0E+05	2.0E-10	1.1

Supplementary Table 3: Affinity data for E09 point mutants and EP6b_B01 revertants. The seven mutations found in the high affinity antibody EP6b_B01 were individually introduced in the parent E09 backbone or removed from EP6b_B01 to measure their impact on affinity improvement. Kinetic parameters and affinity for recombinant Fas of the mutants, revertants, parent E09 and EP6b_B01 were determined by BIAcore. *K*A values (1/*K*D in M-1) were used to calculate free energy of binding compared to the parent E09 ($\Delta\Delta G0$(mut.-WT)) presented in Figure 4B.

Supplementary Table 4

Antibody	Cell killing efficiency (%)	Cell killing EC50 (nM)
E09	75	0.74
EP12r_E01	77	0.90
EP4b_E03	63	0.80
EP5b_E05	43	0.96
EP6b_B01	Non Functional	Non Functional
E09_Y58W	59	2.69
E09_Y52aR	75	1.82
E09_R52bS	34	0.63

Supplementary Table 4: Cell killing data for E09 and variant antibodies. Cell killing efficiency and EC_{50} were determined from *in vitro* viability assays using Jurkat cells and a titration of the anti-Fas antibodies without cross-linking.