Supplementary Results: Skowera et al

Unconventional processing and presentation of preproinsulin signal peptide epitopes

In light of the fact that processing and presentation of PPI_{15-24} is TAP independent, and proteasomal digestion fails to generate SP substrates from PPI_{1-30} with the appropriate COOH terminus, we considered alternative pathways for processing of preproinsulin SP epitopes. It is likely that cleavage of the COOH-terminus of both PPI_{17-24} and PPI_{15-24} epitopes (residue 24) is mediated, at least in part, by signal peptidase (SPase) activity, since this is the point at which preproinsulin is cleaved to yield proinsulin (**Figure 2c**). Indeed, using a synthetic peptide representing the first 41 amino acids of preproinsulin and a preparation of proteins solubilised from human liver ER membranes, we confirmed that soluble ER protease(s), presumed to include SPase, cleave at residue 24. Moreover, soluble ER proteases also cleave at residue 17, thus generating intact PPI_{17-24} (**Supplementary Figure 3a**). In contrast, in the same experiments, PPI_{15-24} could not be visualized (**Supplementary Figure 3b**), suggesting that intact PPI_{15-24} cannot be derived by soluble ER proteases. Using hydrophobicity plots, residue 15 of preproinsulin is predicted to lie within the ER membrane (**Figure 2c**) and is therefore likely to require an intramembrane cleaving protease (I-CLiP). This is an emerging family of enzymes (S1) that require distinct intramembrane topology for optimal function, one of the best characterised being signal peptide peptidase (SPP) (S2). In humans, SPP generates signal-peptide-derived ligands for the non-classical HLA-E molecule (S3) and is involved in processing of the core protein of hepatitis C virus (S4).

However, using RNA-mediated interference designed to knock-down SPP (S5), or the SPP inhibitor $(Z\text{-}LL)_2$ ketone (S2), as strategies to reduce expression of SPP in K562-

PPI-A2.1 cells had minimal effects on PPI_{15-24} presentation as detected using CD8 T-cell clones specific for PPI_{15-24} (data not shown). Future studies will be required to address the nature of the I-CLiP activity that generates PPI_{15-24}. In summary, these results provide evidence that the processing of SP epitopes of preproinsulin is unconventional, requiring SPase and I-CLiP activity, but neither TAP nor proteasomal cleavage.

Supplementary Methods

Processing of PPI_{1-41} by solubilised microsomal membrane proteins was examined using a modified procedure of Weihofen *et al* (S6). Human liver microsomal membranes (Sigma) were resuspended by homogenisation in solubilisation buffer (50 mM Tris-HCl pH 7.8, 50 mM potassium acetate, 2 mM magnesium acetate, 125 mM sucrose, 1 mM DTT, 0.8% CHAPS) and incubated for 30 minutes on ice. Insoluble material was then removed by ultracentrifugation (1 hour 135,000 x *g*) and 100µl solubilised membrane protein incubated with synthetic PPI_{1-41} peptide at 75µM at 30°C for 1 hour followed by centrifugation (3 hours, 15,000 x *g*, 4°C) through a Microcon 3000 MWCO to isolate small peptide fragments. The ultrafiltrate was loaded into Oasis MCX cartridges (Waters) after dilution to 1 ml in water/0.1% TFA, washed at 1 ml/min for 8 minutes, eluted in 1ml 5% ammonium hydroxide/ 30% methanol on ice, and vacuum-concentrated to 100µl before RP-HPLC fractionation and MS analysis as described above.

The possibility that SPP was involved in generation of preproinsulin-SP epitopes was explored in two ways. First, SPP expression was knocked down in K562-PPI-A2 cells using three different SPP and control (EGFP) short hairpin RNA constructs as described (S5) (kind gift of Dr HL Ploegh, Harvard Medical School, MA, USA), achieving robust reduction in SPP levels (data not shown). Reduced SPP or control (EGFP) K562-PPI-A2 cells were then used to stimulate PPI_{15-24}-specific T-cells followed by measurement of intracellular TNF-α production as described above. In addition, SPP activity in K562-PPI-A2 cells was suppressed through culture in the presence of the inhibitor $(Z\text{-}LL)_2$ ketone (S6) (Merck Biosciences, Nottingham, UK) at 1-50µM for 48 hours with daily replenishment, followed by stimulation of PPI_{15-24}-

specific T-cells as described above and measurement of intracellular TNF-α production.

References

S1. Brown, M.S., Ye, J., Rawson, R.B., and Goldstein, J.L. 2000. Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* 100:391-398.

S2. Weihofen, A., Binns, K., Lemberg, M.K., Ashman, K., and Martoglio, B. 2002. Identification of signal peptide peptidase, a presenilin-type aspartic protease. *Science* 296:2215-2218.

S3. Bland, F.A., Lemberg, M.K., McMichael, A.J., Martoglio, B., and Braud, V.M. 2003. Requirement of the proteasome for the trimming of signal peptide-derived epitopes presented by the nonclassical major histocompatibility complex class I molecule HLA-E. *J Biol Chem* 278:33747-33752.

S4. McLauchlan, J., Lemberg, M.K., Hope, G., and Martoglio, B. 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *Embo J* 21:3980-3988.

S5. Loureiro, J., Lilley, B.N., Spooner, E., Noriega, V., Tortorella, D., and Ploegh, H.L. 2006. Signal peptide peptidase is required for dislocation from the endoplasmic reticulum. *Nature* 441:894-897.

S6. Weihofen, A., Lemberg, M.K., Ploegh, H.L., Bogyo, M., and Martoglio, B. 2000. Release of signal peptide fragments into the cytosol requires cleavage in the transmembrane region by a protease activity that is specifically blocked by a novel cysteine protease inhibitor. *J Biol Chem* 275:30951-30956.

Supplementary Figure Legends

Supplementary Figure 1

Phenotypic characterization of preproinsulin signal peptide epitope specific CD8 T cell clone. (a) Surface expression of markers of differentiation, activation, migration and co-stimulation are shown as flow cytometry histograms in which lines represent isotype control staining and shaded regions represent staining for the marker indicated. Representative data are shown for clone PPI_{15-24}-specific T cell clone 1E6, tested under resting conditions. (b) Surface and intracellular staining of granule proteins, cytokines and effector molecules that may have a role in CTL killing potential. Representative data are shown for PPI_{15-24}-specific T cell clone 1E6, tested under resting and activated (with PPI_{15-24}-pulsed APCs) conditions as indicated.

Supplementary Figure 2

CD8 T cell clones specific for preproinsulin signal peptide PPI_{15-24} are cytotoxic and kill human beta cells in a glucose-regulated fashion. Upper panel shows percent specific lysis of human HLA-A2+ (open squares) islet cells by PPI_{15-24}-specific T-cell clones 3F2, 2D6 and 1C8. HLA-A2-negative islet cells (triangles) are not killed. Islets were maintained in 5mM glucose throughout. Experiments were performed in triplicate and shown as means with error bars representing SEMs.

Lower panel shows percent specific lysis of human islet cells exposed to 14 hours of pre-culture at different glucose concentrations (5.6mM, 11mM and 20mM glucose concentrations are represented by filled squares, circles and open squares, respectively) by CTL clones 3F2 and 1C8. Higher glucose concentrations result in significantly enhanced CTL killing.

Supplementary Figure 3

The role of ER proteases in generation of PPI_{15-24} and PPI_{17-24}. MS analyses of selected RP-HPLC fractions from digests of PPI_{1-41} in the presence of soluble preparations of human liver microsomal membranes were conducted, showing that (a) PPI_{17-24} (784.42 m/z) can be generated intact by soluble ER proteases. In contrast (b), the epitope PPI_{15-24} (predicted mass 968.4836 m/z; expected location indicated by arrow) is not observed in the expected RP-HPLC fraction shown here, or in any adjacent fractions (data not shown), indicating that the NH_2-terminal of this peptide cannot be generated by soluble ER proteases.

SUPPLEMENTARY Table 1. Interferon-γ ELISPOT response to preproinsulin peptides in HLA-A2+ Type 1 diabetes patients and HLA-A2+ non-diabetic control subjects

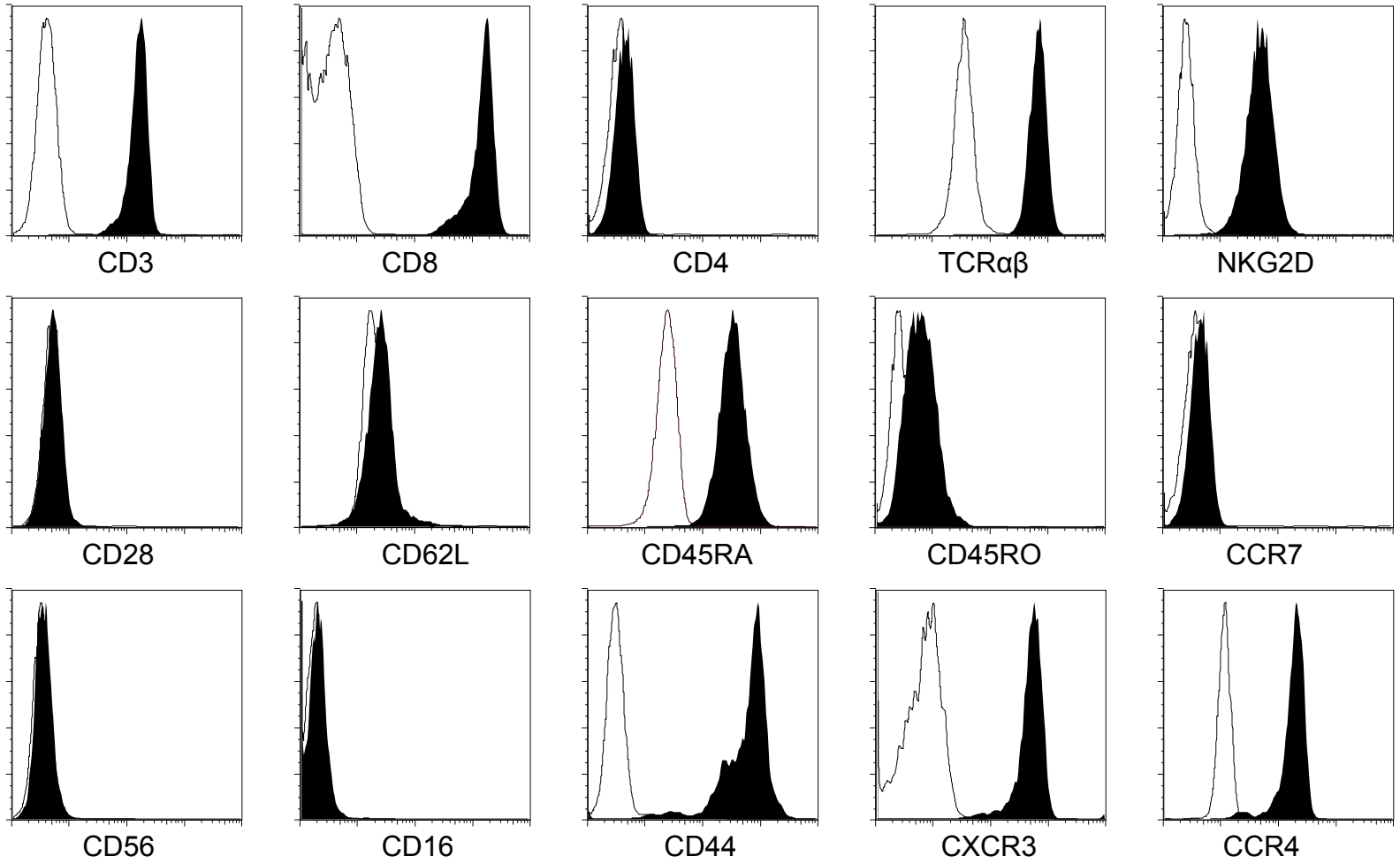
Clinical details			Raw counts (spots/million PBMCs)				Stimulation index		
Case	Age	Sex	Bckgd	CEF[1]	PPI_{17-24}	PPI_{15-24}	CEF[1]	PPI_{17-24}	PPI_{15-24}
PATIENTS									
1	34	M	12	18	11	22	1.5	0.9	1.8
2	32	M	15	65	7	5	4.3	0.5	0.3
3	33	M	5	99	9	9	19.8	1.8	1.8
4	27	F	2	37	10	2	18.5	5.0	1.0
5	31	M	5	105	7	9	21.0	1.4	1.8
6	21	M	5	38	5	12	7.6	1.0	2.4
7	28	M	11	359	7	5	32.6	0.6	0.5
8	41	F	2	14	3	3	7.0	1.5	1.5
9	34	M	1	22	8	9	22.0	8.0	9.0
10	21	F	1	14	4	1	14.0	4.0	0.0
11	37	F	4	81	5	15	20.3	1.3	3.8
12	29	M	2	43	3	3	21.5	1.5	1.5
13	33	M	9	45	36	26	5.0	4.0	2.9
14	33	M	1	284	5	4	284.0	5.0	4.0
15	36	F	3	162	12	11	54.0	4.0	3.7
16	18	F	12	422	15	29	35.2	1.3	2.4
17	36	M	11	65	58	39	5.9	5.3	3.5
18	35	M	17	262	22	20	15.4	1.3	1.2
19	31	F	5	15	0	1	3.0	0.0	0.2
20	37	M	2	40	4	8	20.0	2.0	4.0
21	40	M	2	32	49	21	16.0	24.5	10.5
22	26	M	16	159	11	16	9.9	0.7	1.0
23	32	M	17	262	48	54	15.4	2.8	3.2
24	25	F	4	738	34	16	184.5	8.5	4.0
25	33	F	23	123	30	62	5.3	1.3	2.7
26	36	M	1	55	5	5	55.0	5.0	5.0
27	35	M	1	41	1	1	41.0	1.0	1.0
28	41	M	4	54	12	8	13.5	3.0	2.0
29	21	F	5	50	4	4	10.0	0.8	0.8
30	18	F	29	66	13	23	2.3	0.4	0.8
31	36	M	30	349	11	13	11.6	0.4	0.4
32	23	M	8	479	12	36	59.9	1.5	4.5
33	33	M	35	409	37	44	11.7	1.1	1.3
34	24	F	25	301	28	20	12.0	1.1	0.8
35	33	F	28	40	28	24	1.4	1.0	0.9
36	30	M	1	189	1	1	189.0	1.0	1.0
37	26	F	7	67	9	14	9.6	1.3	2.0
38	26	F	3	275	5	18	91.7	1.7	6.0
39	24	M	4	4	5	2	1.0	1.3	0.5
40	31	M	5	99	7	18	19.8	0.8	3.6
41	32	F	10	51	9	18	5.1	0.9	1.8
CONTROLS									
1	36	F	7	25	7	5	3.6	1.0	0.7
2	39	M	42	265	29	31	6.3	0.7	0.7
3	27	F	14	16	13	6	1.1	0.9	0.4
4	33	F	5	82	1	1	16.4	0.2	0.2

5	37	F	3	146	8	1	48.7	2.7	0.3
6	37	F	1	155	1	1	155.0	1.0	1.0
7	30	F	8	66	5	9	8.3	0.6	1.1
8	26	M	13	177	19	17	13.6	1.5	1.3
9	26	M	7	19	1	16	2.7	0.1	2.3
10	28	M	31	109	36	30	3.5	1.2	1.0
11	33	F	2	1	2	3	0.5	1.0	1.5
12	29	F	4	25	2	3	6.3	0.5	0.8
13	46	M	15	103	20	19	6.9	1.3	1.3
14	32	M	12	152	15	11	12.7	1.3	0.9
15	38	F	7	115	8	6	16.4	1.1	0.9
16	30	F	7	118	4	6	16.9	0.6	0.9
17	24	M	11	145	15	20	13.2	1.4	1.8
18	42	M	40	115	25	53	2.9	0.6	1.3
19	35	M	7	135	4	4	19.3	0.6	0.6
20	31	M	14	49	18	28	3.5	1.3	2.0
21	42	F	9	435	12	9	48.3	1.3	1.0

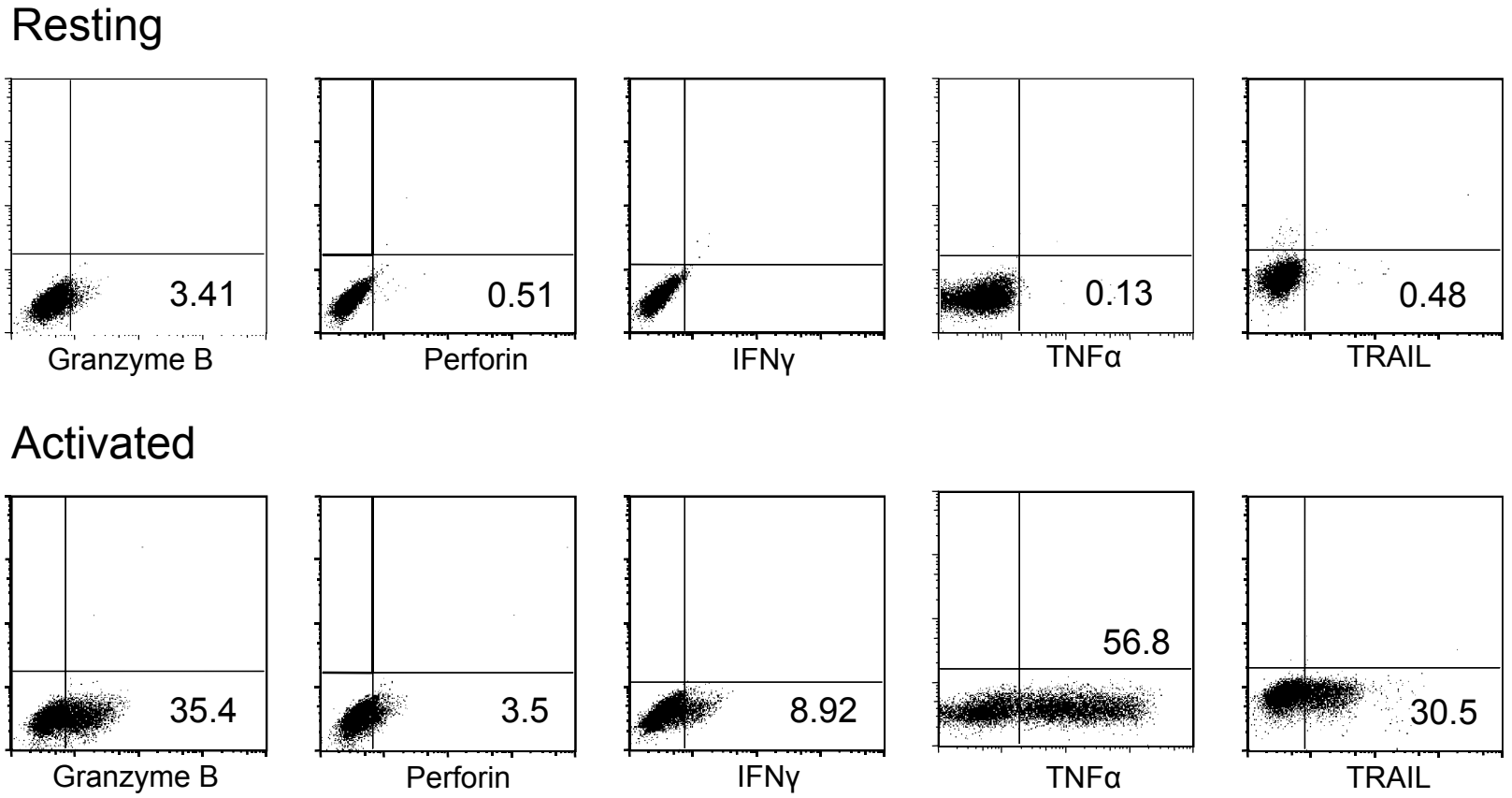
[1]CEF=mix of viral peptides as positive control

Supplementary Figure 1

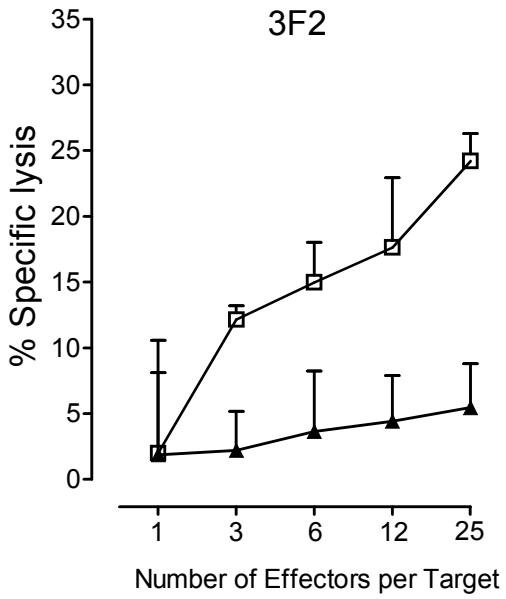
a

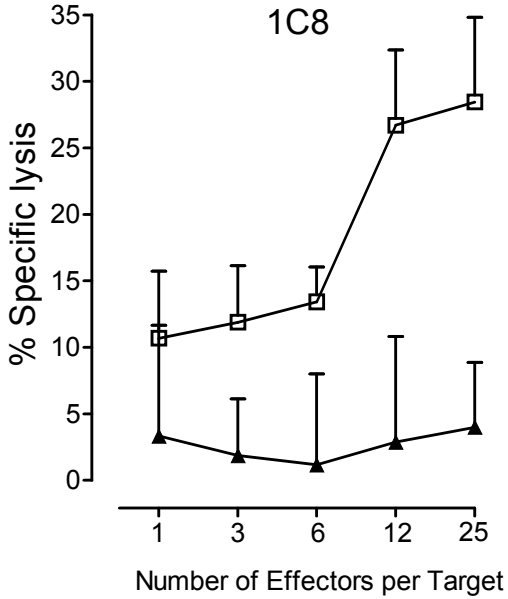


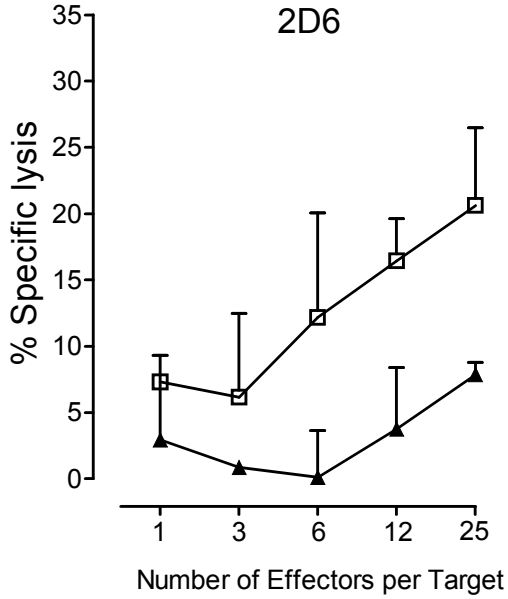
b

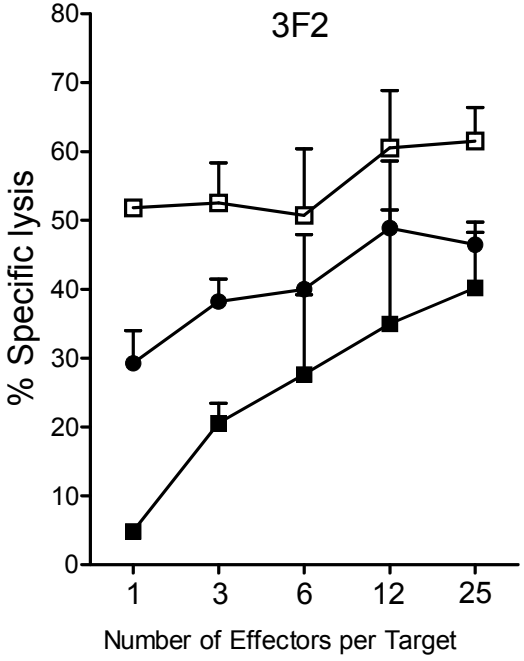

Resting
3.41
Granzyme B
0.51
Perforin
IFNγ
0.13
TNFα
0.48
TRAIL
Activated
35.4
Granzyme B
3.5
Perforin
8.92
IFNγ
56.8
TNFα
30.5
TRAIL

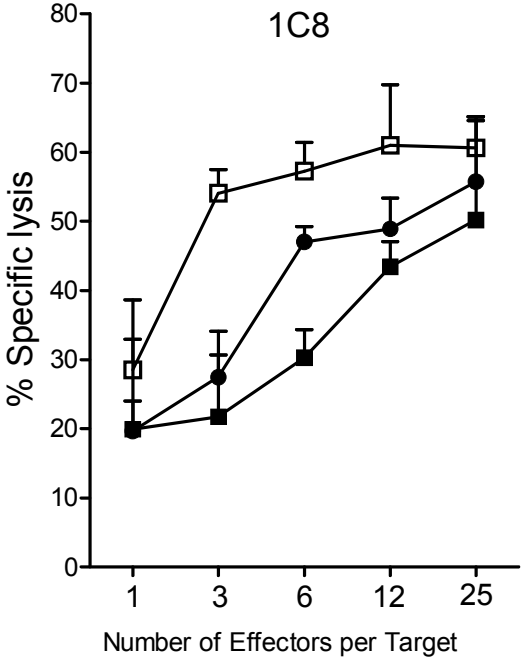
Supplementary Figure 2











Supplementary Figure 3