

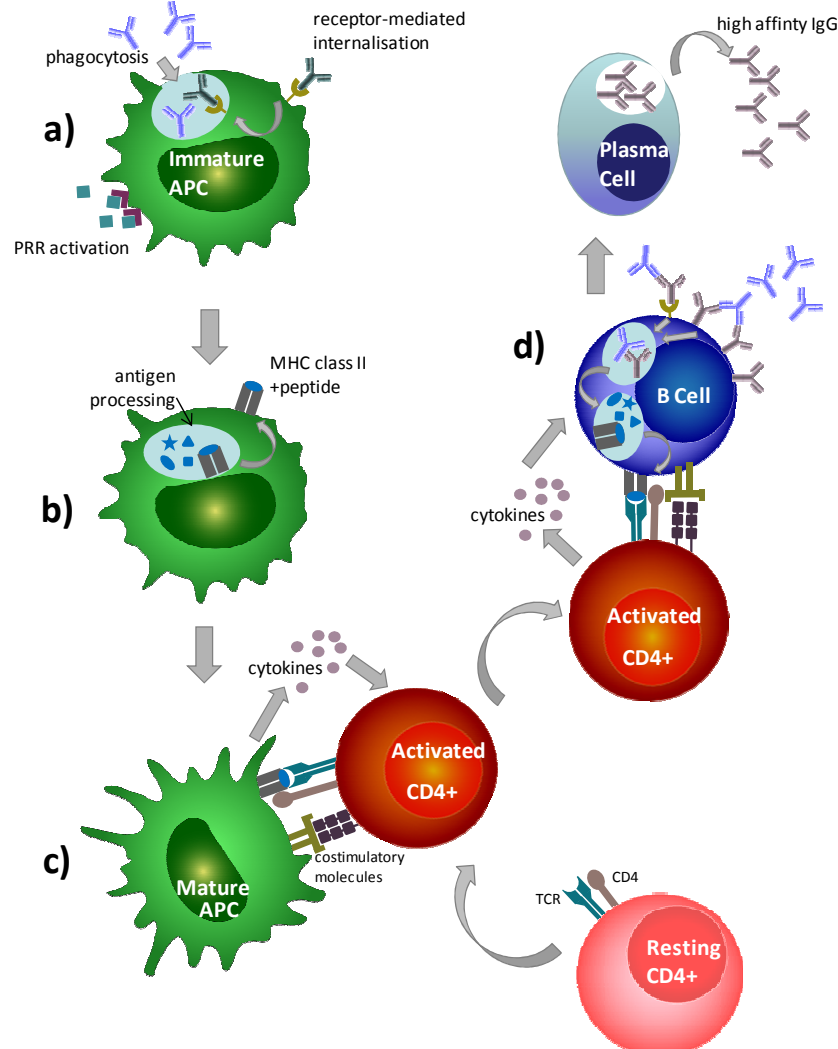


The Effects of Induced Aggregation of IgG and Insulin Preparations on *In Vitro* T Cell Stimulation

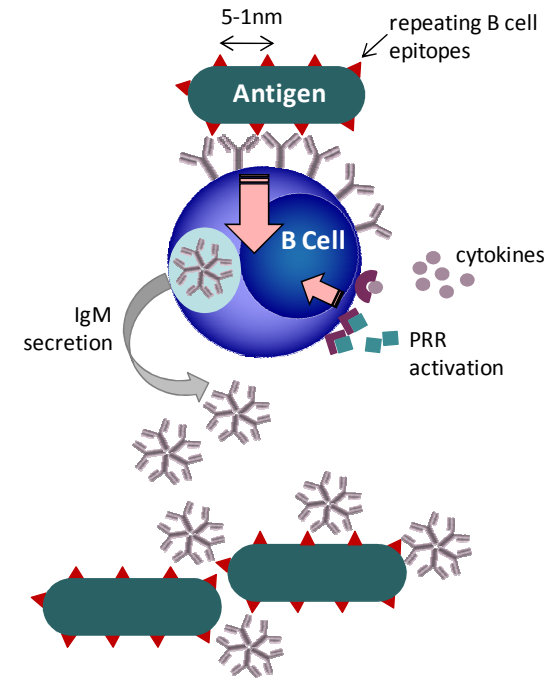
2nd EIP Symposium
17th-19th November 2009

Immune Responses to Protein Therapeutics

T-Dependent

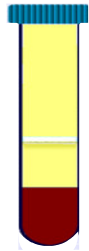


T-Independent

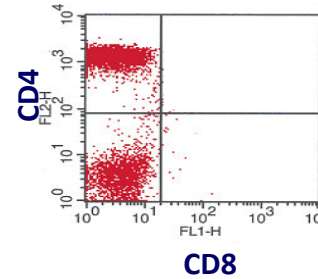
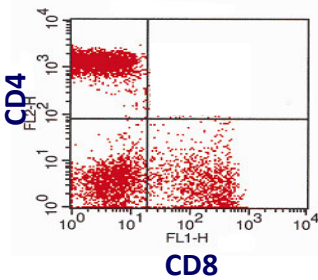


T Cell Epitope Identification using *Ex Vivo* T Cell Assays

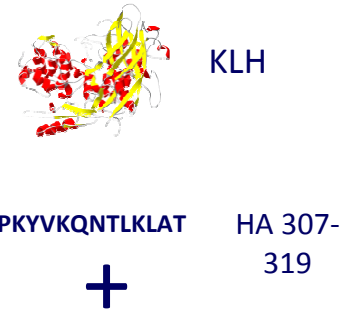
1. Human Blood Sample



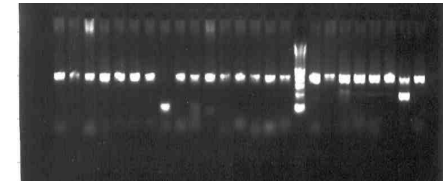
2. T Cell Subset Depletion



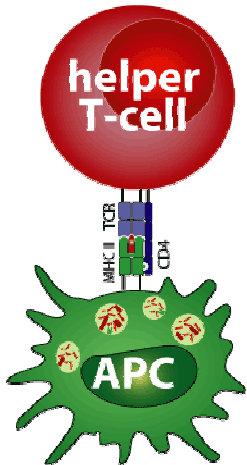
3. Donor Characterization



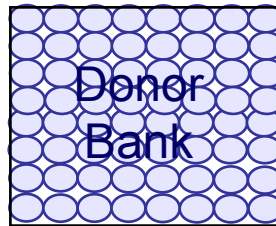
MHC Class II allotyping



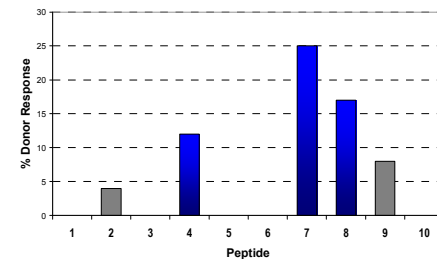
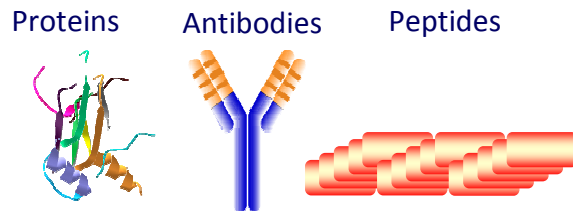
5. Donor Selection



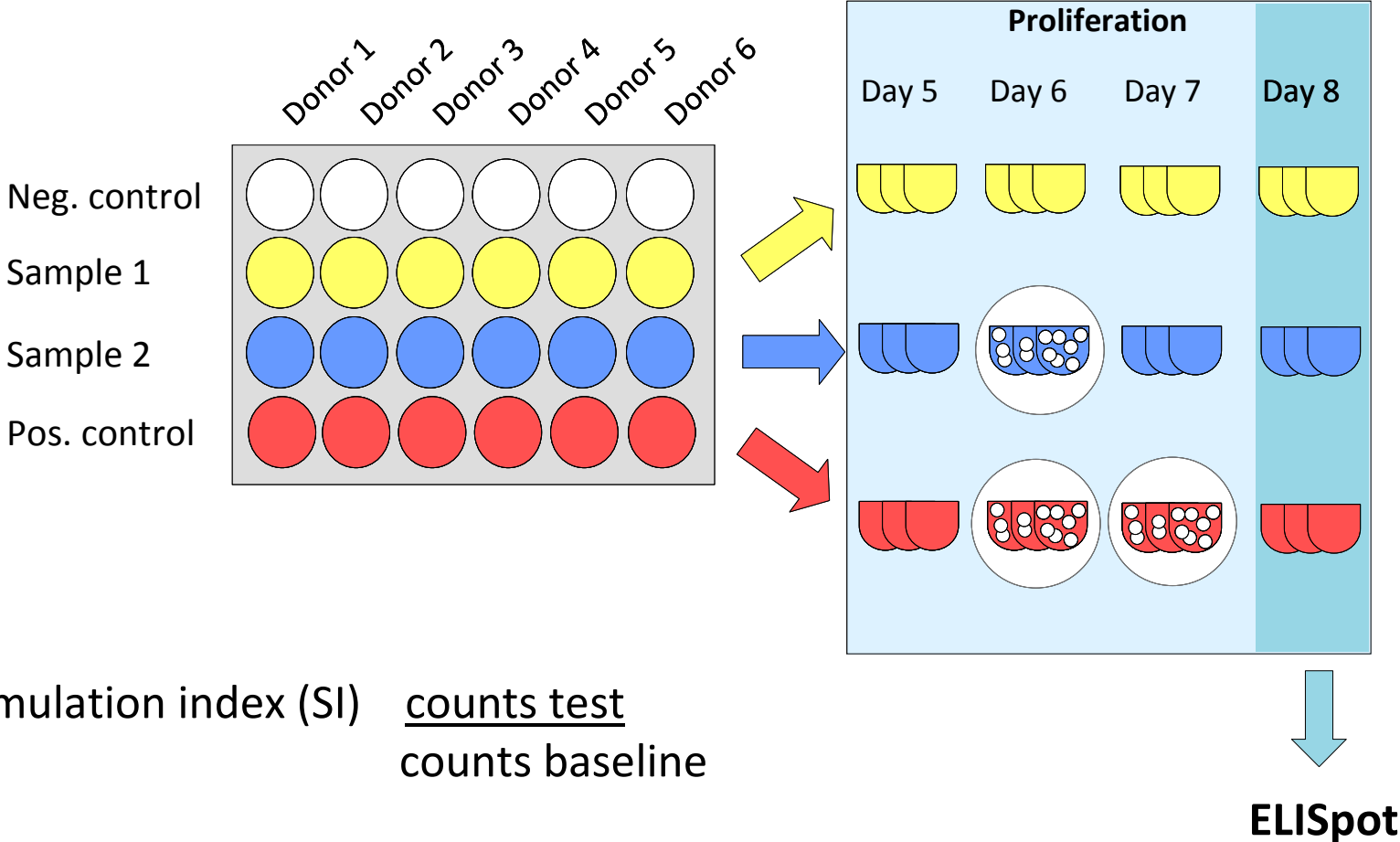
4. Optimized Storage in Liquid Nitrogen



6. Detection of CD4+ T Cell Responses



In Vitro Time Course T Cell Assay



Stimulation index (SI) $\frac{\text{counts test}}{\text{counts baseline}}$

'Positive' response = $SI \geq 2, p < 0.05$

Effects of Induced Aggregation on *In Vitro* T Cell Stimulation

- Humanized IgG1 and rh Insulin
- Induced aggregation (Jiskoot lab);
 - IgG - 5 x freeze thawing
 - IgG – Shaking 500rpm 24 hours
 - Insulin - Gluteraldehyde
 - Insulin - Heating 75°C 1 hour
- Test samples and controls for *in vitro* T cell stimulation
 - 50 donors
 - T cell proliferation/IL-2 secretion
 - Analysis of monocyte derived DC phenotype

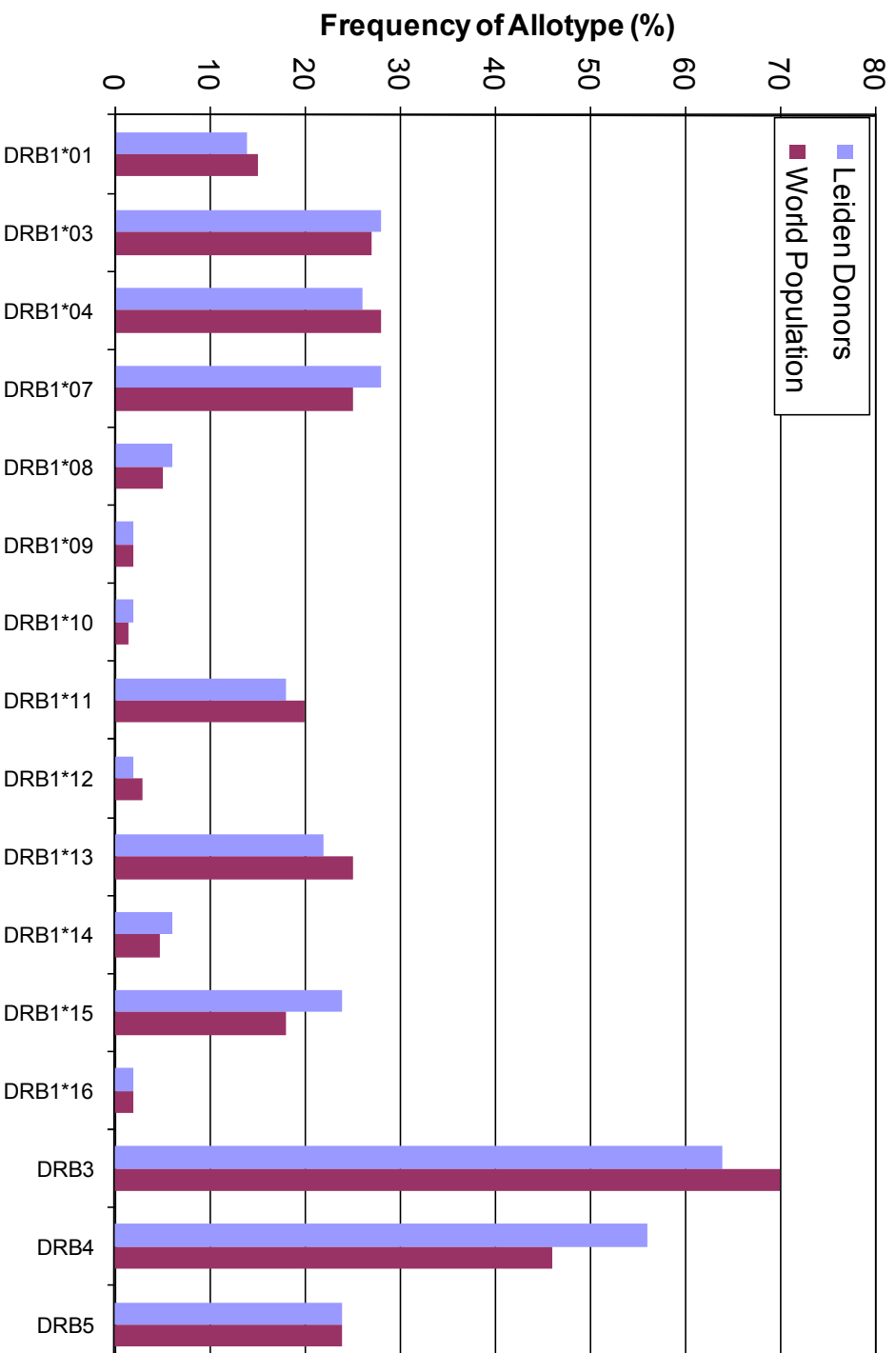
Overview IgG1 Aggregates

Lane	Formulation	Stress conditions	Structural properties	Quantity /Type of aggregates
1	1 mg/mL IgG 100 mM phosphate pH 7.2	5 freeze-thawing cycles	<ul style="list-style-type: none"> • non-covalent aggr. • intact secondary & tertiary structure 	< 0.1% particles larger 1µm 0.2% larger soluble aggr. 1.8% dimers 97.3% monomer
2		Unstressed control	native IgG	1.5% dimers 98.0% monomer
3	1 mg/mL 10 mM citrate 5% (w/v) Sucrose pH 6	Shaking 500 rpm, 24h	<ul style="list-style-type: none"> • mainly non-covalent aggr. • loss of secondary and tertiary structure • hydrophobic aggr. 	14% large insoluble aggr. 0.5% soluble aggr. 1.3% dimer/trimer 84% monomer
4		Unstressed control	native IgG	1.5% dimers 98.0% monomer

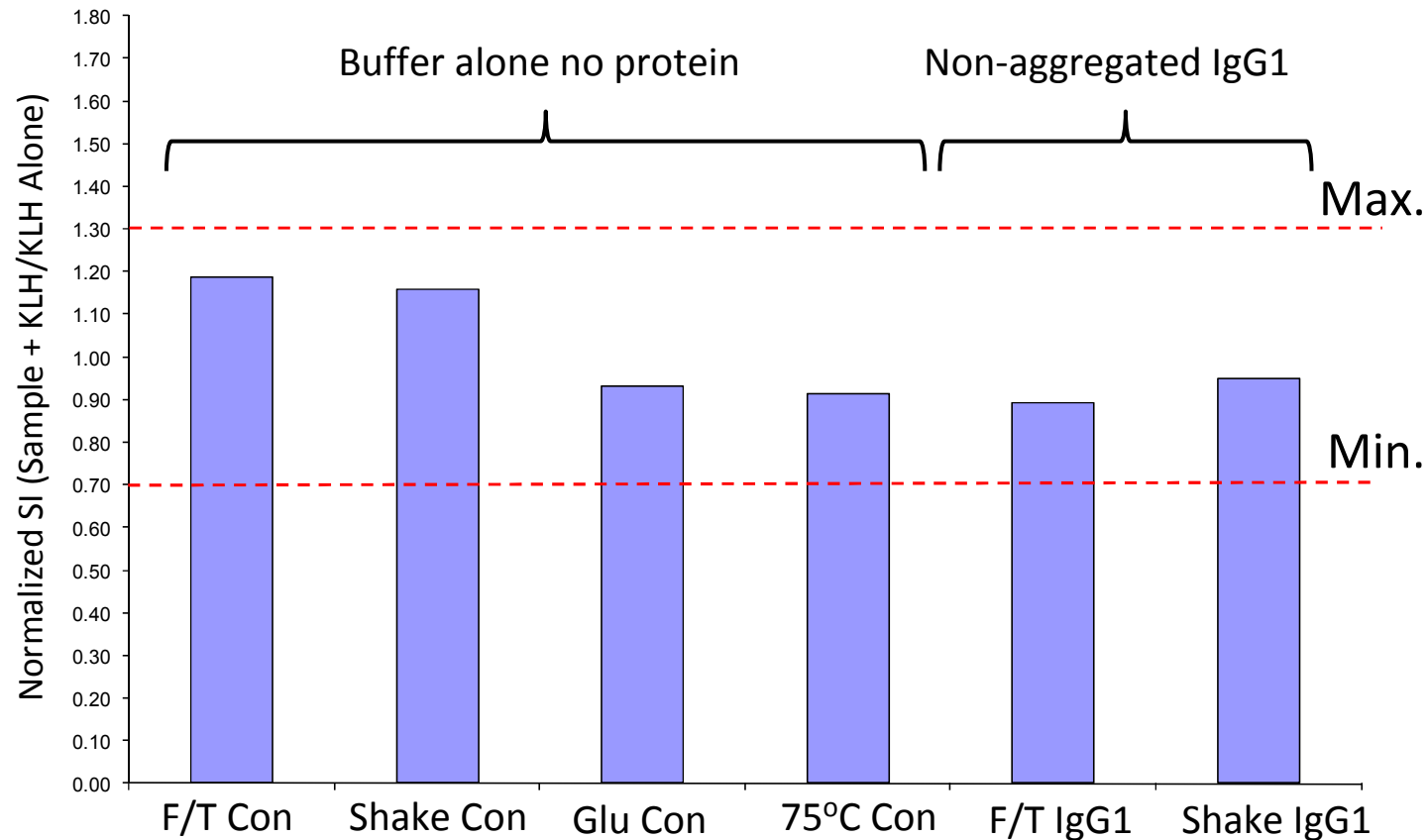
Overview Insulin Aggregates

Lane	Formulation	Stress conditions	Characteristic of the samples	Quantity /Type of aggregates
5	1 mg/mL insulin 50 mM phosphate pH 7.4	Glutar-aldehyde crosslinking	<ul style="list-style-type: none"> • covalent soluble aggregates • intact epitopes • loss of secondary and tertiary structure 	59% sol. aggregates 17% dimer 24% monomer
6		Unstressed insulin control	native insulin	
7	1 mg/mL insulin 69 mM Na ₂ HPO ₄ 45 mM NaCl 57 μM ZnCl ₂ 29 μM CaCl ₂ , pH 7.4	Heating : 1 h 75°C	<ul style="list-style-type: none"> • covalent insoluble aggregates • epitopes not intact • loss of secondary and tertiary structure 	54% insol. aggregates 14% sol. aggregates 10% dimer 22% monomer
8		Unstressed insulin control	native insulin	

Selection of Donor Haplotypes



Buffers and Humanized IgG do not Directly Modulate *In Vitro* T Cell Activation



- 10 donors selected known to respond against KLH
- Data shown mean SI normalised data for 10 donors (sample plus KLH vs KLH alone)

In Vitro T Cell Stimulation with Aggregates

Donor No	IgG	IgG	IgG	IgG	Insulin	Insulin	Insulin	Insulin	KLH	KLH
	F/T	Con F/T	Shake	Con Shake	Glu	Con Glu	75oC	75oC Con	Fresh	Study
1									4.01	4.05
2			+		+		+	+	4.14	1.44
3			+		+				7.77	6.54
4	+		+		+				2.52	7.88
5			+						4.47	7.50
6	+		+						5.44	25.52
7									1.48	0.48
8			+				+	+	21.20	7.12
9									5.91	18.83
10									6.87	11.78
11									2.32	2.18
12			+	+					23.02	36.97
13									2.76	12.07
14	+		+		+	+		+	5.21	4.64
15									18.84	7.63
16					+				4.07	4.38
17									4.34	6.82
18									21.63	20.26
19									1.90	7.73
20									2.41	6.60
21									3.18	4.33
22									3.09	10.72
23									1.00	9.12
24									4.87	3.66
25			+						8.09	12.90
26							+		6.46	5.13
27			+						0.56	1.83
28			+						3.26	26.44
29									3.03	6.21
30					+	+		+	9.76	3.38
31	+	+	+	+			+	+	1.39	0.91
32									20.35	4.92
33									2.95	9.02
34									1.81	1.57
35									2.44	3.28
36									30.55	3.20
37									23.11	3.40
38			+						3.48	1.93
39									20.55	2.49
40			+						17.24	4.89
41									8.72	4.94
42									10.53	6.10
43									3.19	2.87
44									3.70	9.03
45									4.15	7.73
46									2.79	1.97
47									4.00	2.32
48									1.21	1.58
49			+						37.59	13.23
50	+	+	+	+			+	+	5.26	2.16

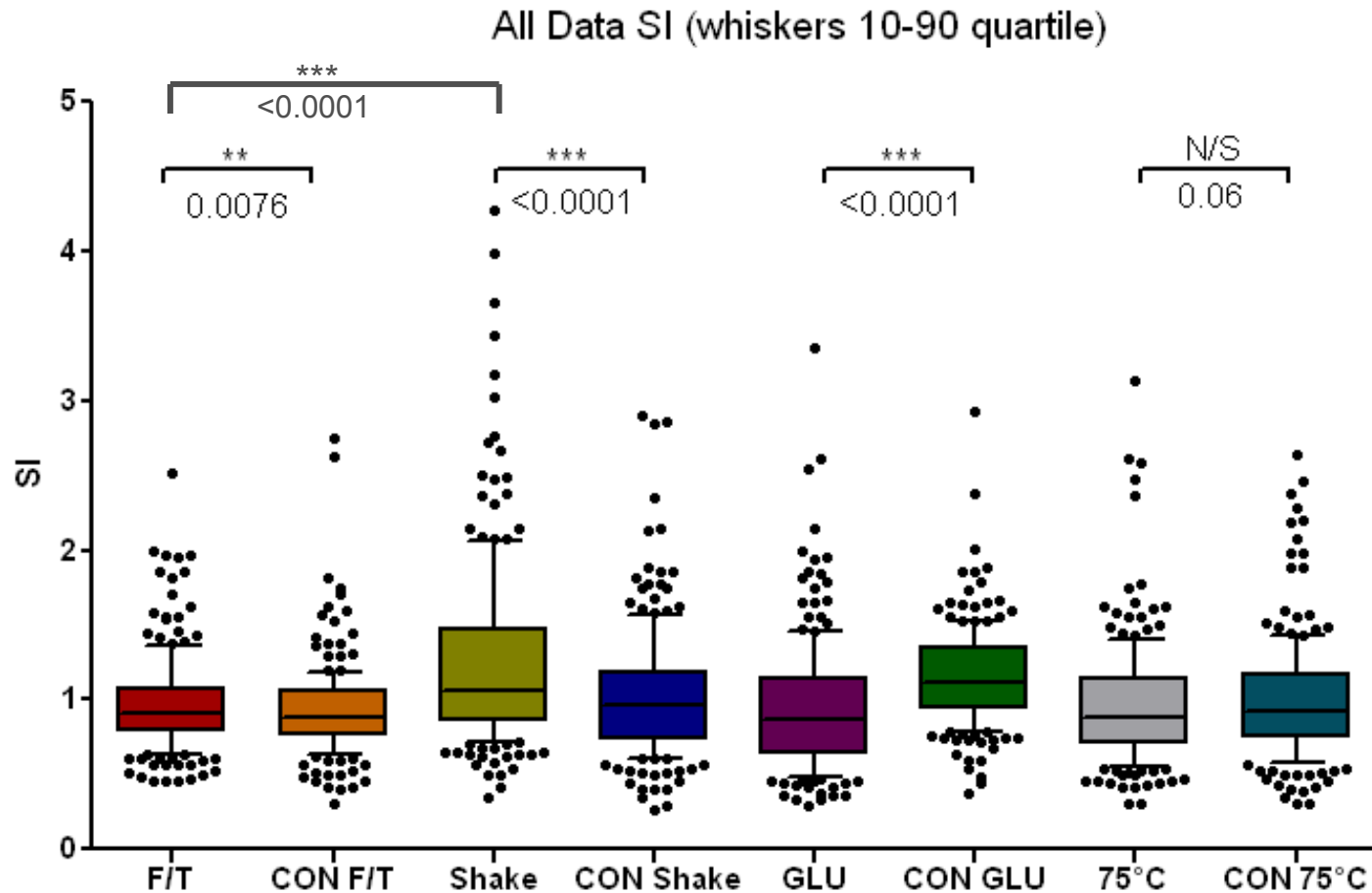
	IgG			
	F/T	Con F/T	Shake	Con Shake
Proliferation	10%	4%	32%	6%
IL-2	8%	6%	24%	10%
Both	8%	4%	24%	6%

	Insulin			
	Glu	Con Glu	75oC	75oC Con
Proliferation	12%	4%	10%	12%
IL-2	16%	4%	10%	8%
Both	12%	0%	10%	6%

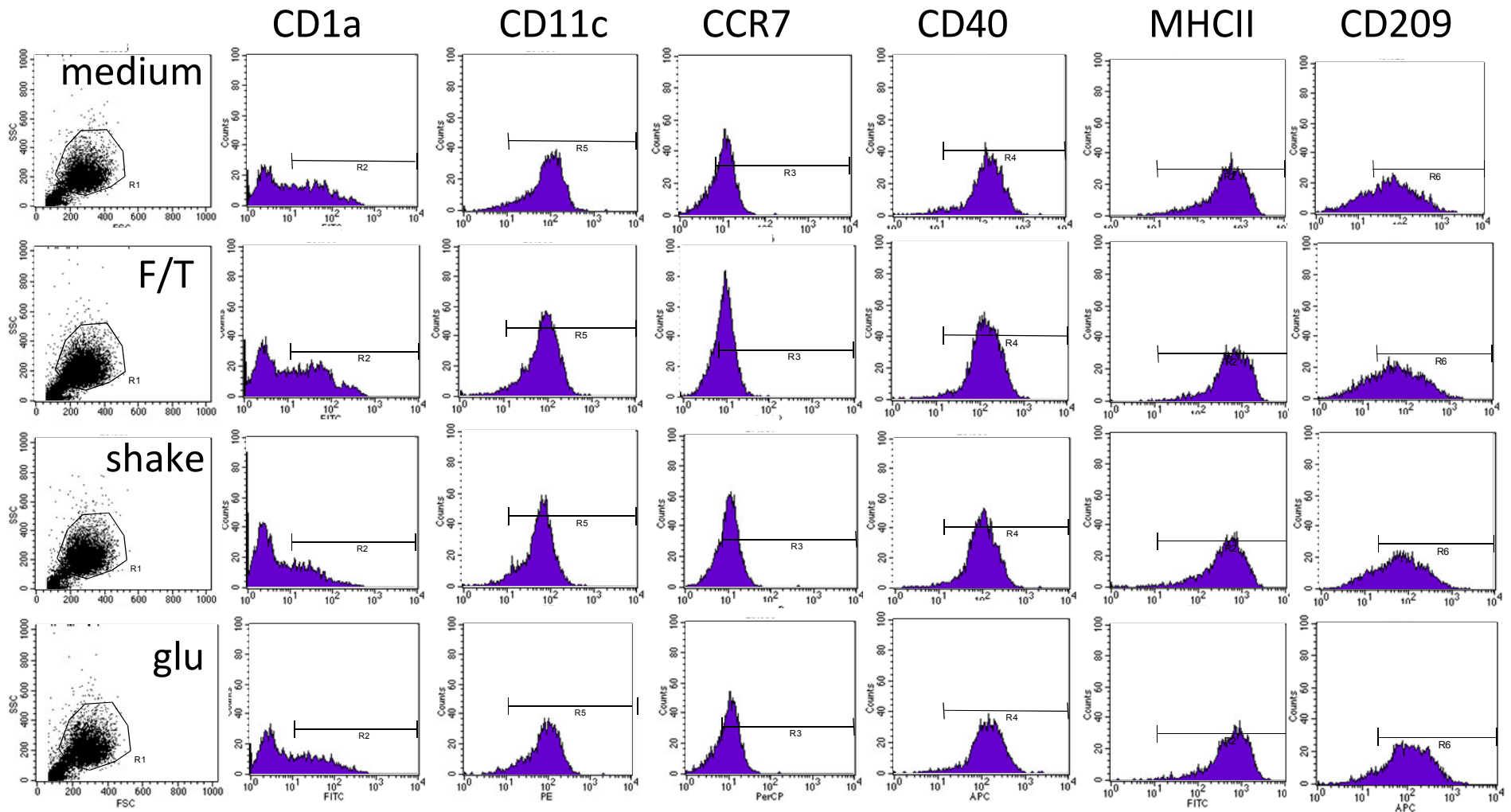
SI₂, p<0.05 (cpm or spw test vs baseline)

- IL-2 ELISpot
- + Proliferation

Analysis of all Donor Responses



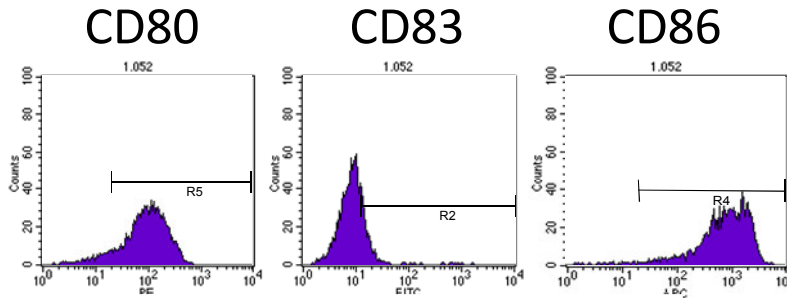
In Vitro Matured Mo Derived DC



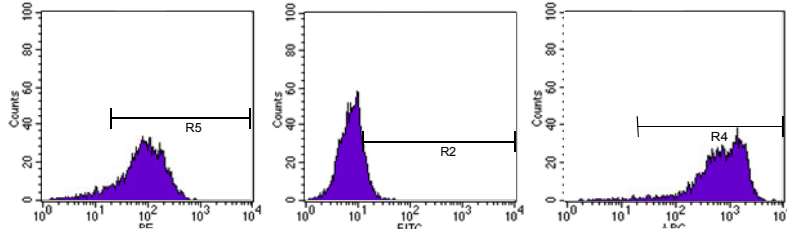
Mature (IL-4/GM-CSF/TNF α) DC Phenotypic Analysis

In Vitro Matured Mo Derived DC

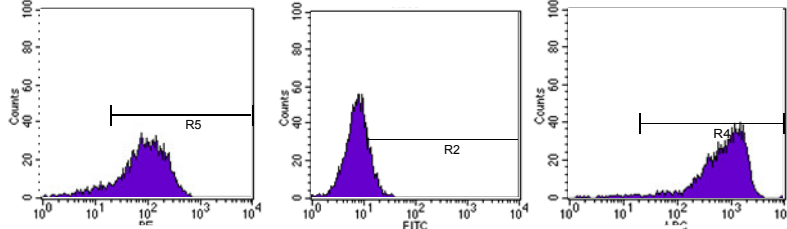
medium



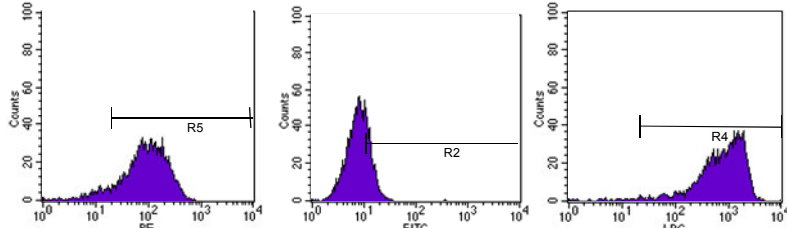
F/T



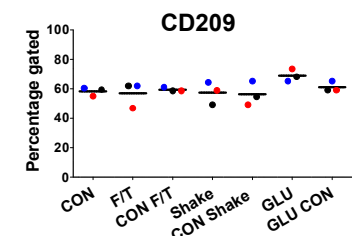
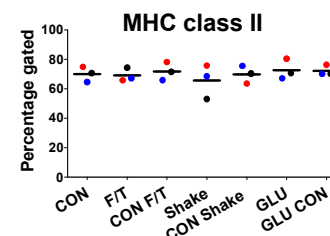
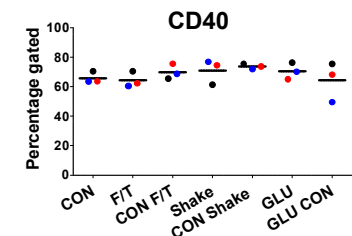
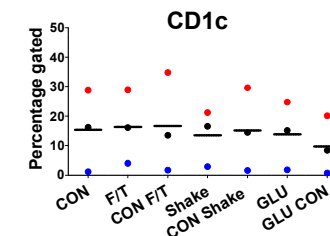
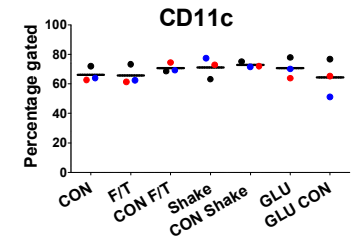
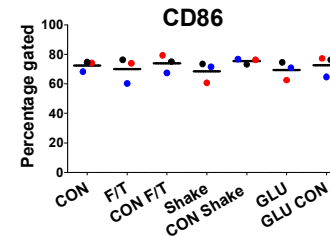
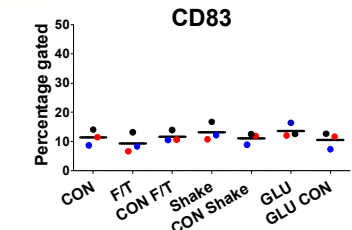
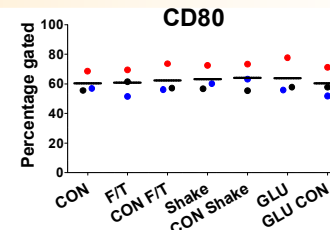
shake



glu



Mature (IL-4/GM-CSF/TNF α) DC Phenotypic Analysis



Summary

- Protein aggregates can enhance *in vitro* T cell stimulation.
- Enhanced stimulation may be restricted to aggregates with specific properties e.g. size, quantity and/or physiochemical properties.
 - Aggregates induced by shaking IgG1 induced more frequent and higher magnitude T cell responses than aggregates induced by F/T.
 - Large insoluble aggregates and/or loss of structure?
- T cell tolerance to insulin may influence *in vitro* responses against induced insulin aggregates an effect that is not necessarily observed with humanized IgG1 (containing engineered V regions).
- Aggregates do not alter Mo derived DC phenotype after maturation *in vitro*.
- Possible effects due to aggregate induced DC licensing, increased protein uptake = more effective presentation to T cells (Ho *et al* 2008, Immunity)?

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