Influence of Aggregates on *In Vitro* T Cell Responses

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Antitope

EIP Tuesday 7th February

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Aggregation of Protein Therapeutics

- Highly undesirable; decreased biological activity of the protein, and the potential to trigger adverse immune responses.
- Enhanced immune responses to protein aggregates have been reported in animal and clinical studies (*van Beers, Pharma. Res. 2011, Moore and Leppert J. Clin. endocrin. 1980*).
- Aggregates displaying non-native protein conformations may be seen by the immune system as neoantigens and facilitate breaking of tolerance which could trigger antibody formation.
- Characteristic adaptive humoral immune response requiring T cell activation.

Investigation into Effects of Protein Aggregates on T Cell Immune Responses

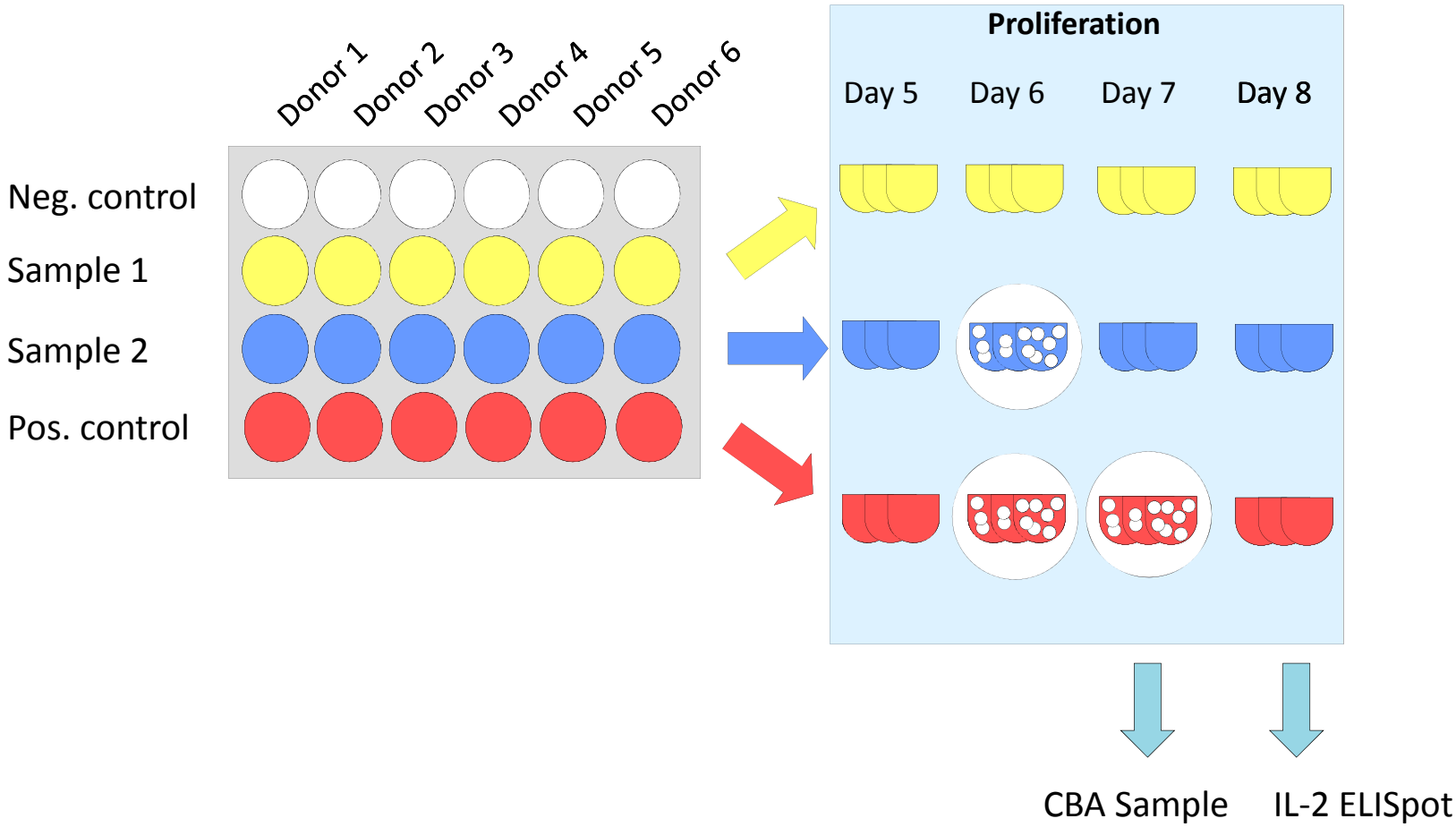
- Induce protein therapeutics to aggregate using variety of ‘stress’ conditions.
- Compare ability of different aggregate samples to induce in vitro T cell responses.
- Aggregates produced with different properties:
    - % aggregation in sample
    - Soluble/insoluble
    - Affect structure i.e. covalent (loss of 2^{o} and/or 3^{o} structure)
    - Small <1µm or large >10µm (typically hydrophobic)

Study 1:
In Vitro T Cell Activation Induced by Human IgG and Human Insulin Aggregates

- PBMC (depleted of CD8+ T cells) from 50 healthy donors stimulated with aggregate samples and T cell activation measured.
- IgG antibody induced to aggregate under stress conditions:
  - Shaking 24h at 500rpm
  - 5 cycles of freeze thawing
- Insulin aggregates induced under stress conditions:
  - Glutaraldehyde cross linking
  - Heat denaturation

Collaboration: Dr Jiskoot, University Leiden

In Vitro EpiScreen Time Course T Cell Assay: CBA, Proliferation and ELISpot



Study 1:
Overview Generation of IgG1 Aggregates

ID	Formulation	Stress conditions	Structural properties	Quantity /Type of aggregates
F/T	1 mg/mL IgG 100 mM phosphate pH 7.2	5 freeze-thawing cycles	• non-covalent aggr. • intact secondary & tertiary structure	< 0.1% particles larger 1µm 0.2% larger soluble aggr. 1.8% dimers 97.3% monomer
Control F/T		Unstressed control	native IgG	1.5% dimers 98.0% monomer
Shake	1 mg/mL 10 mM citrate 5% (w/v) Sucrose pH 6	Shaking 500 rpm, 24h	• 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
Control Shake		Unstressed control	native IgG	1.5% dimers 98.0% monomer

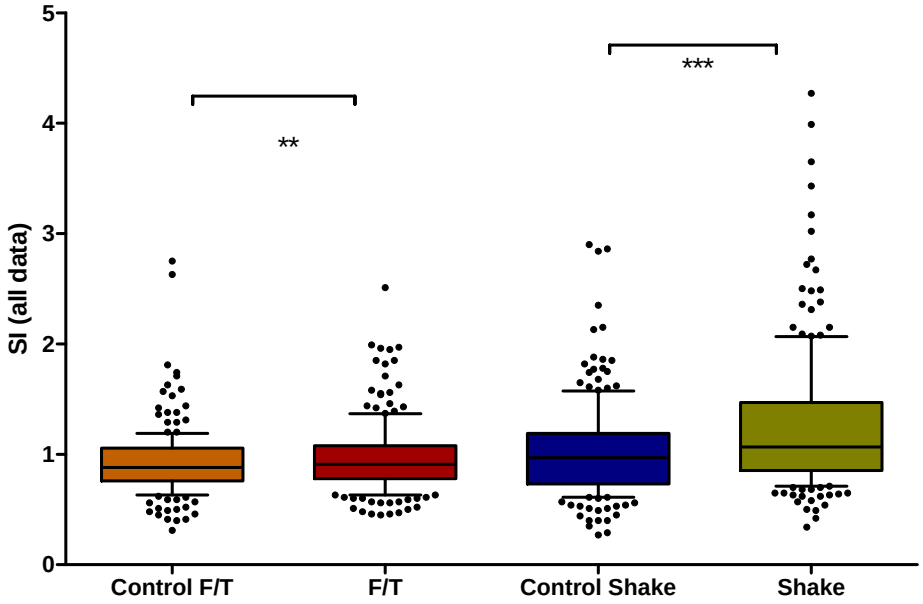
Study 1:
In Vitro T Cell Stimulation with IgG1 Aggregates

IgG1 → Induced to Aggregate → Test in EpiScreen

Treatment 1: F/T = freeze thaw (x5),
Treatment 2: Shake = shaking 500rpm/24hr

50 healthy donors
Time course T cell assay

Magnitude of Response



Box Whiskers (Whiskers 10-90 percentile)

Frequency of Positive Responses (%)

SI≥2.00, p<0.05 (cpm or spw test vs baseline)

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

2 fold

4 fold

Study 1:
In Vitro T Cell Stimulation with Insulin Aggregates

Frequency Positive Responses

SI≥2.00, $p<0.05$ (cpm or spw test vs baseline)

Frequency	Control Glu	Glu	Control Heat	Heat
Proliferation	4%	16%	6%	12%
IL-2	4%	16%	8%	16%
Both	2%	16%	6%	12%

8 fold

2 fold

Insulin Study ⇨ Glutaraldehyde Stress
High frequency soluble aggregates

IgG Study ⇨ Shaking Stress (IgG Study)
High frequency large insoluble aggregates

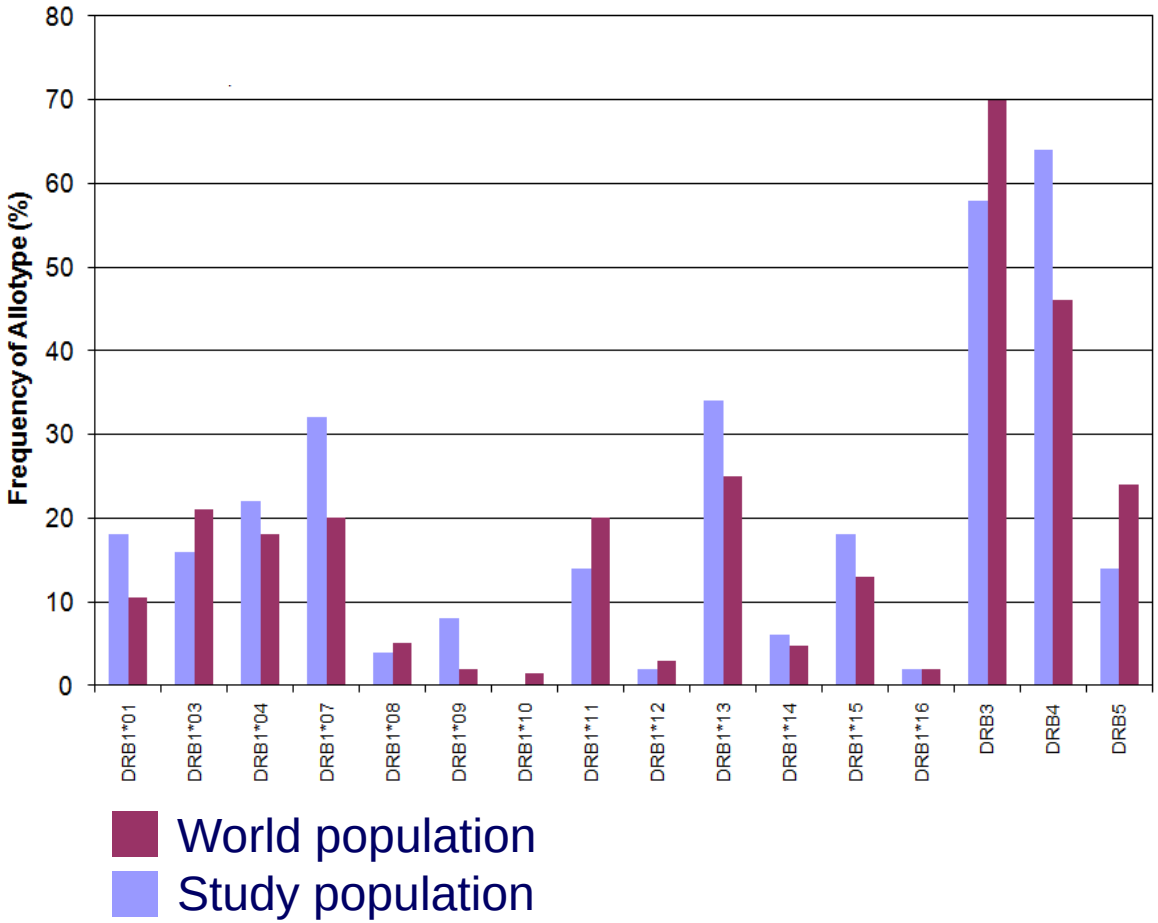
⇨ Loss of tertiary structure

Study 2:
Humira and Herceptin Aggregates

- Samples comprised of monomeric or aggregated clinical antibodies.
- Compare aggregated samples of antibodies associated with high and low risk of clinical immunogenicity in *in vitro* T cell assays.
- Test samples for T cell immunogenicity using EpiScreen time course T cell assay against cohort of 50 donors.
- Measure T cell activation by proliferation, IL-2 ELISpot.
- Determine relative risk of immunogenicity.

Study 2:
Donor Selection

50 healthy donors



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Study 2:
In Vitro T Cell Assay Data (Proliferation and IL-2)

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Donor 9	Donor 10	Donor 11	Donor 12	Donor 13	Donor 14	Donor 15	Donor 16	Donor 17	Donor 18	Donor 19	Donor 20	Donor 21	Donor 22	Donor 23	Donor 24	Donor 25	Donor 26	Donor 27	Donor 28	Donor 29	Donor 30	Donor 31	Donor 32	Donor 33	Donor 34	Donor 35	Donor 36	Donor 37	Donor 38	Donor 39	Donor 40	Donor 41	Donor 42	Donor 43	Donor 44	Donor 45	Donor 46	Donor 47	Donor 48	Donor 49	Donor 50
Herceptin																																					PE*														
Agg. Herceptin	PE*		PE	E	P*E	PE	PE	PE	P			PE	PE					PE	E						P						PE	P*E		PE		PE	PE				PE		PE	PE	E*			PE	PE	PE
Humira					PE	PE	PE		PE									PE		P*E													PE						P*	PE							PE		
Agg. Humira	PE*	PE*	P*E*		PE	PE	PE	PE		PE*					P*					PE		PE*								PE		PE*	PE	P*E							PE			PE*					E
KLH	PE		PE	PE	PE	PE	PE	PE	PE	PE	PE	PE	PE	PE		PE*	E	PE	PE	PE	PE	PE	PE	PE*	PE		E	PE	PE	E	E	P	PE	PE		PE		PE			PE*	E	PE	PE	PE	PE	P*	E	PE	P

P = Positive Proliferation Response

E = Positive ELISpot Response

Frequency of T cell responses (%)

SI≥2.00, $p<0.05$ (cpm or spw test vs baseline)

	Proliferation %	ELISpot %	Proliferation and ELISpot %
Herceptin	2	2	2
Agg. Herceptin	44	46	40
Humira	22	20	20
Agg. Humira	34	34	32
KLH	74	80	68

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1.5 fold

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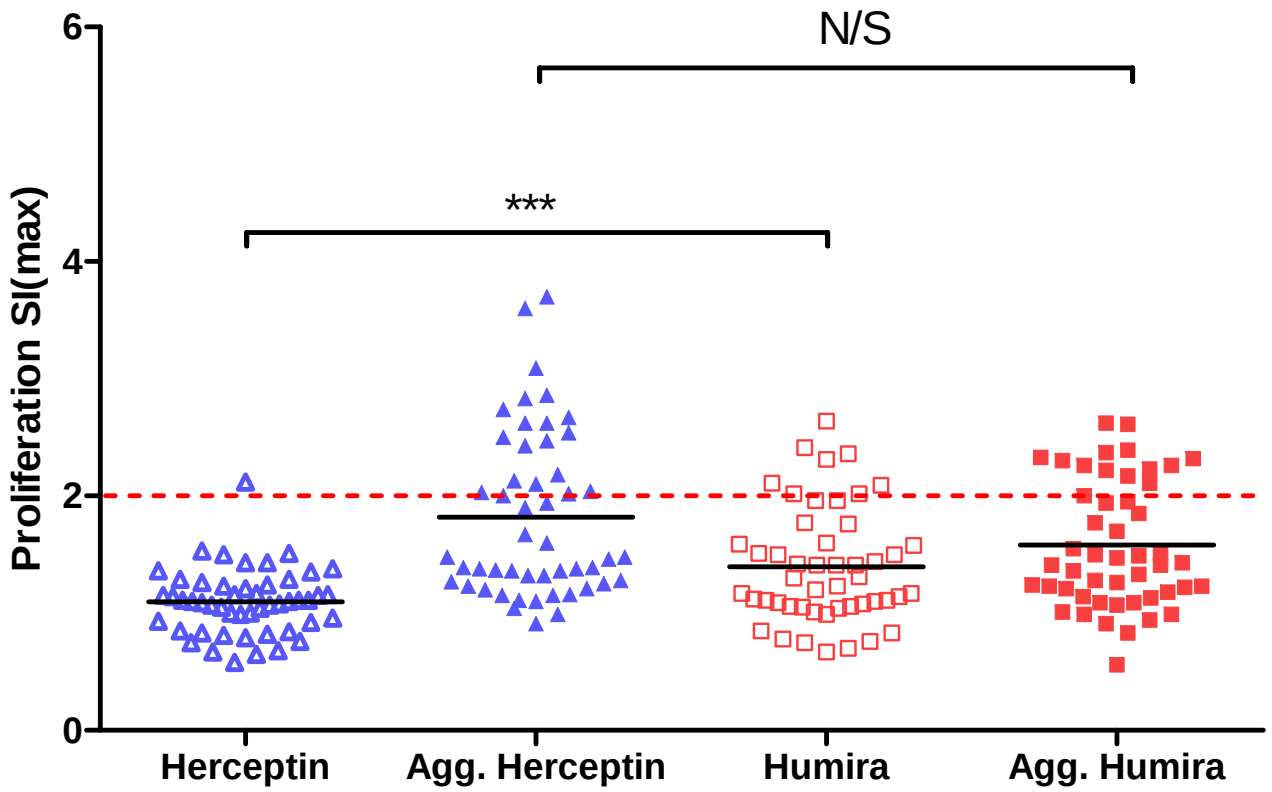
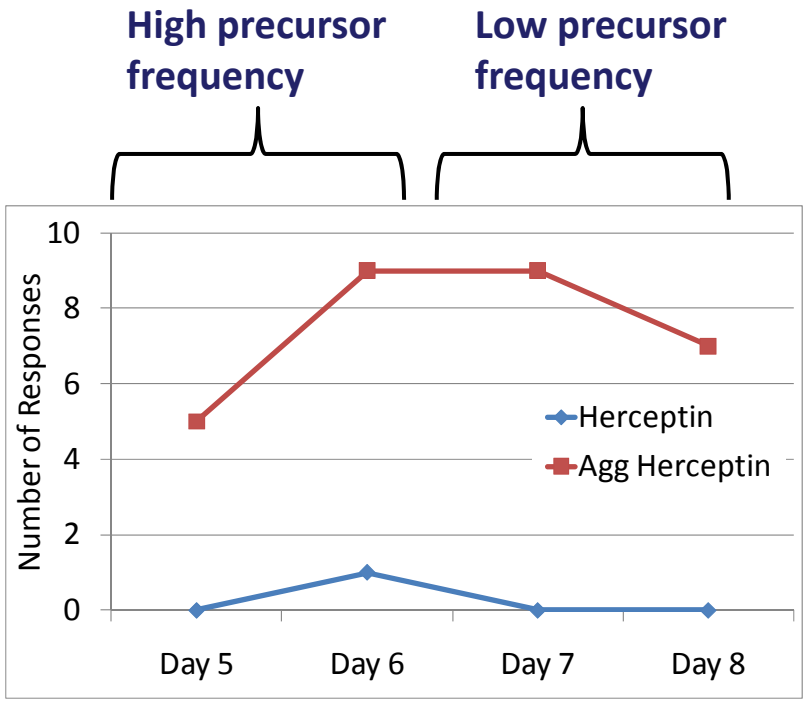
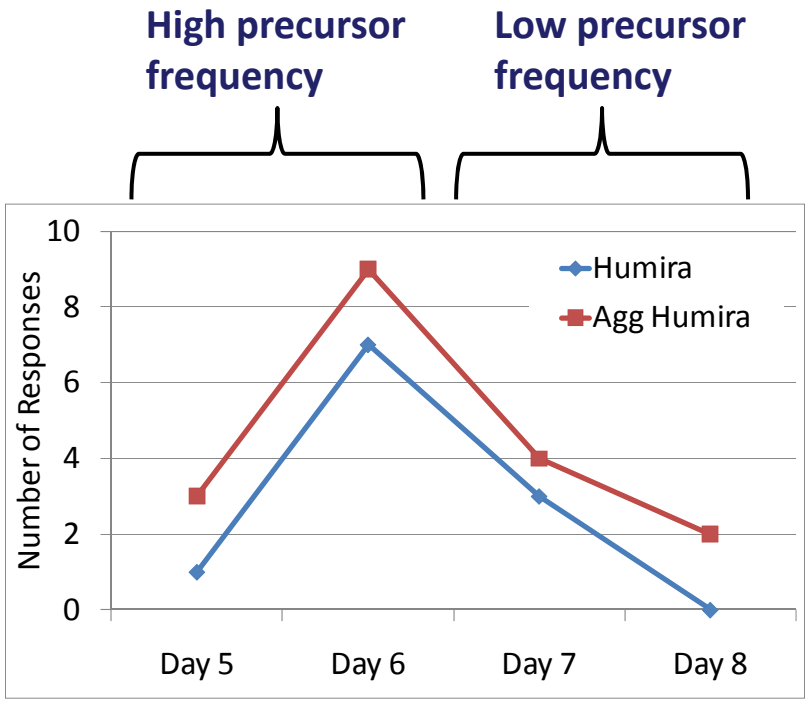
Study 2:
Aggregation Induces High Magnitude T Cell Responses



Study2:
T Cell Precursor Against Aggregated Antibody



Herceptin



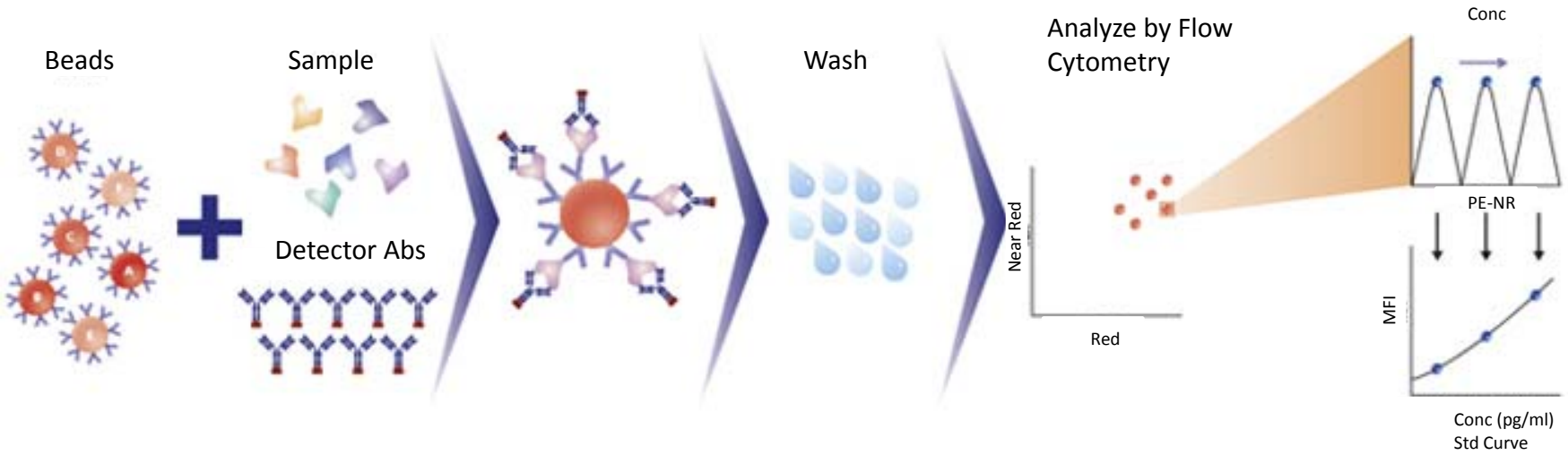
Humira

Study 1 and 2: Conclusions

- Aggregates of antibodies and other protein therapeutics enhance in vitro T cell responses.
- Antibodies that show different levels of clinical immunogenicity (Humira and Herceptin) have a similar capacity to activate T cells when induced to aggregate.
- The kinetics of T cell proliferation indicate that aggregates can induce rapid T cell (polyclonal?) responses.

→ Mechanism of enhanced T cell responses with aggregates, is likely to include enhanced activation of DC: Possibly via PRR, leading to increased expression of co-stimulatory molecules and proinflammatory cytokine secretion.

Characterisation of Aggregate Induced T Cell Response by Cytometric Bead Array (CBA)



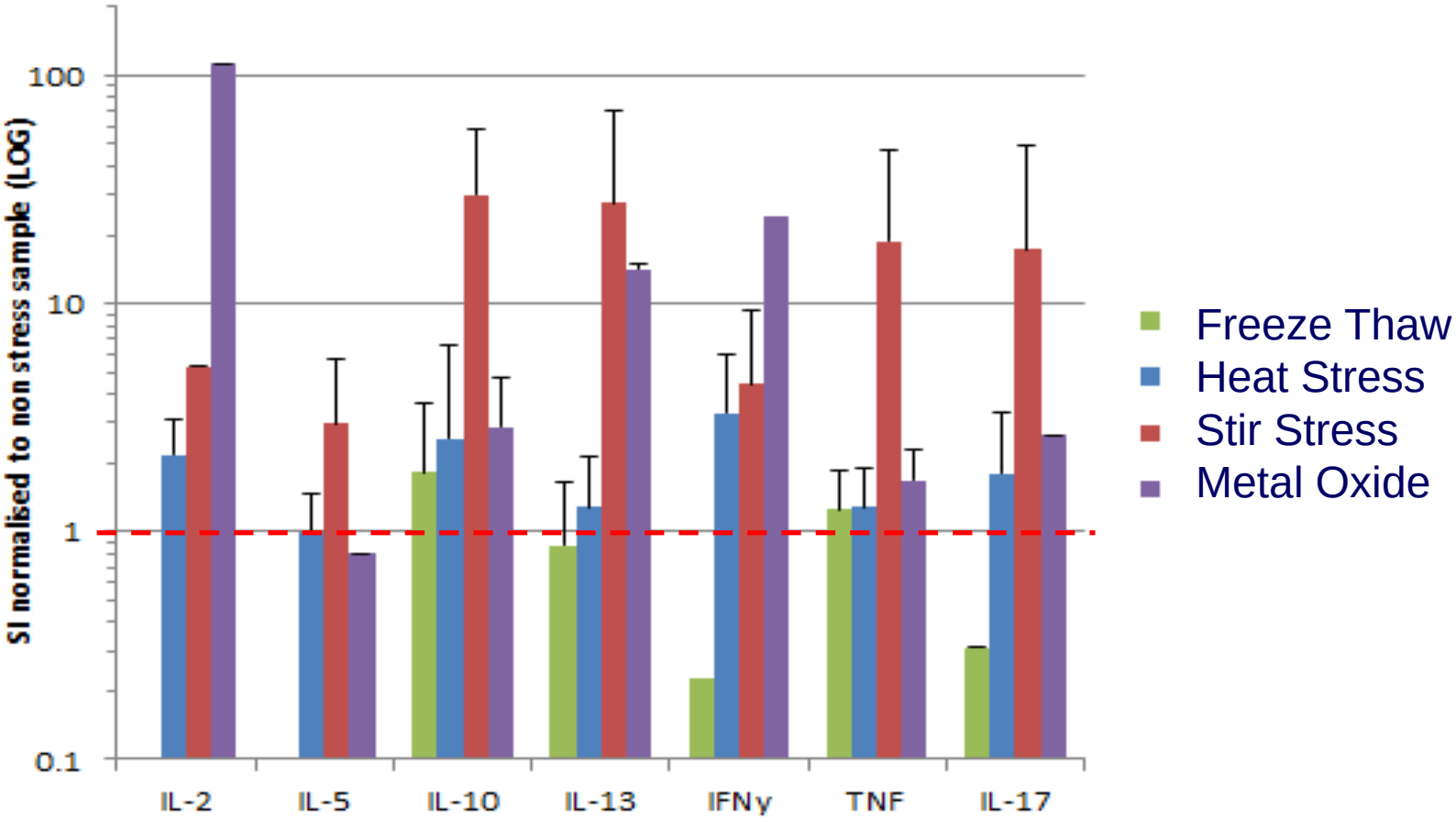
1. Capture beads have a unique fluorescence intensity and are coated with a capture antibody specific for a single cytokine.
2. A combination of different beads is mixed with a sample or standard + mixture of detection antibodies that are conjugated to a reporter molecule (PE).
3. Samples are acquired on a flow cytometer.
4. Standard curves are generated and test sample concentrations determined.

Study 3: Cytokine Secretion from Aggregate Treated $CD8^+$ Depleted PBMC and DC

- Cytokine secretion from aggregate and monomer stimulated PBMC and aggregate stimulated MØ-derived dendritic cells
- Characterised aggregates of a clinical antibody: Stir, freeze thaw, and heat stress.
- Included metal catalyst oxidation. Strong responses in animal models (*van Beers 2011, Hermeling 2005 Pharma. Res)* .
- Cytokine secretion was measure by multiplex cytokine bead assay.

CB1

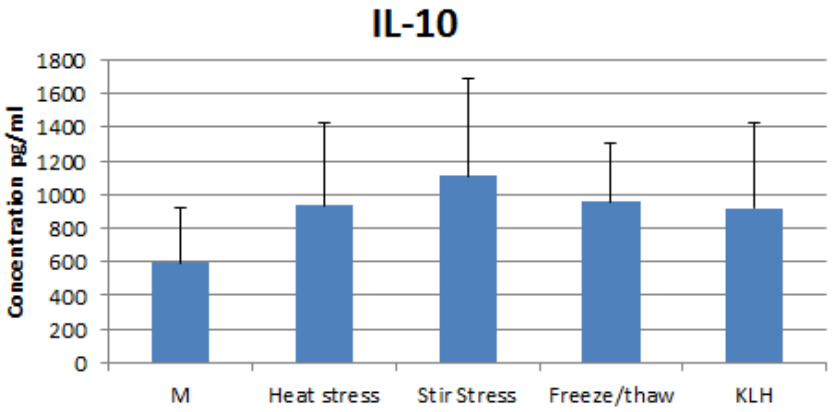
Study 3, Cytokine Profile: Antibody Aggregate Stimulated CD8+ Depleted PBMC

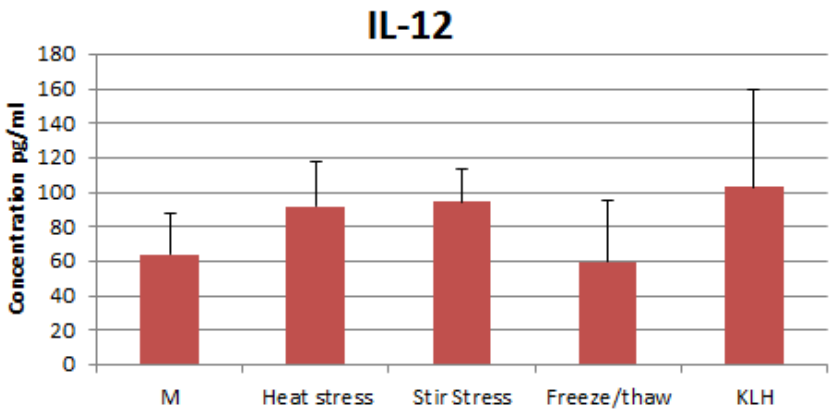


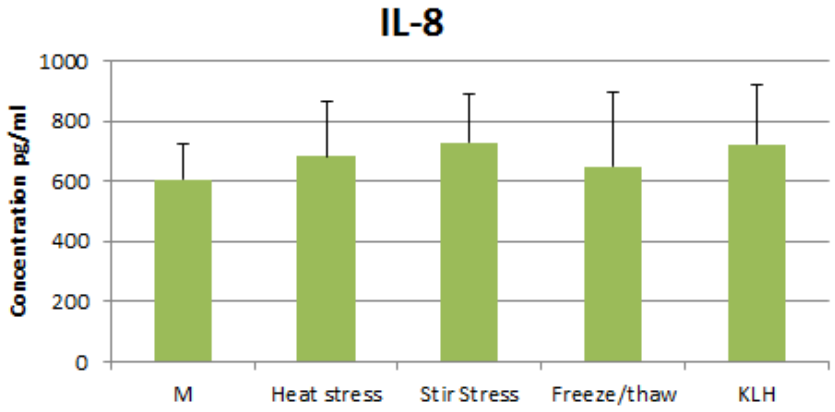
Slide 17

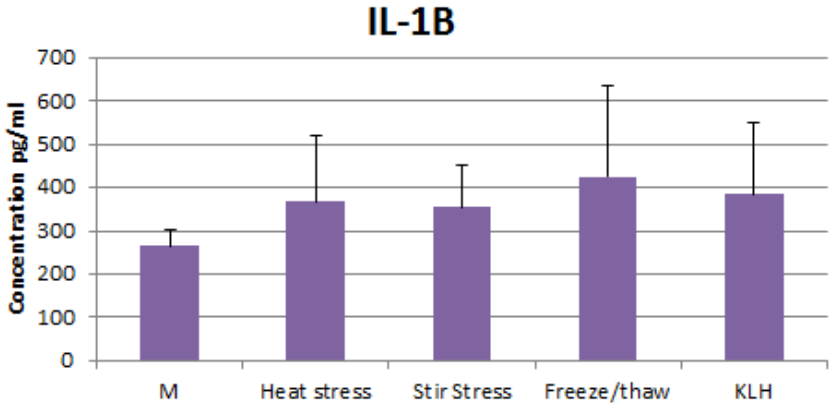
CB1 Will probably take out MO. Will speak to Andrea first.
Christine Bryson, 2/3/2012

Study 3, Cytokine Profile: Antibody Aggregate Treated MØ-Derived Dendritic Cells





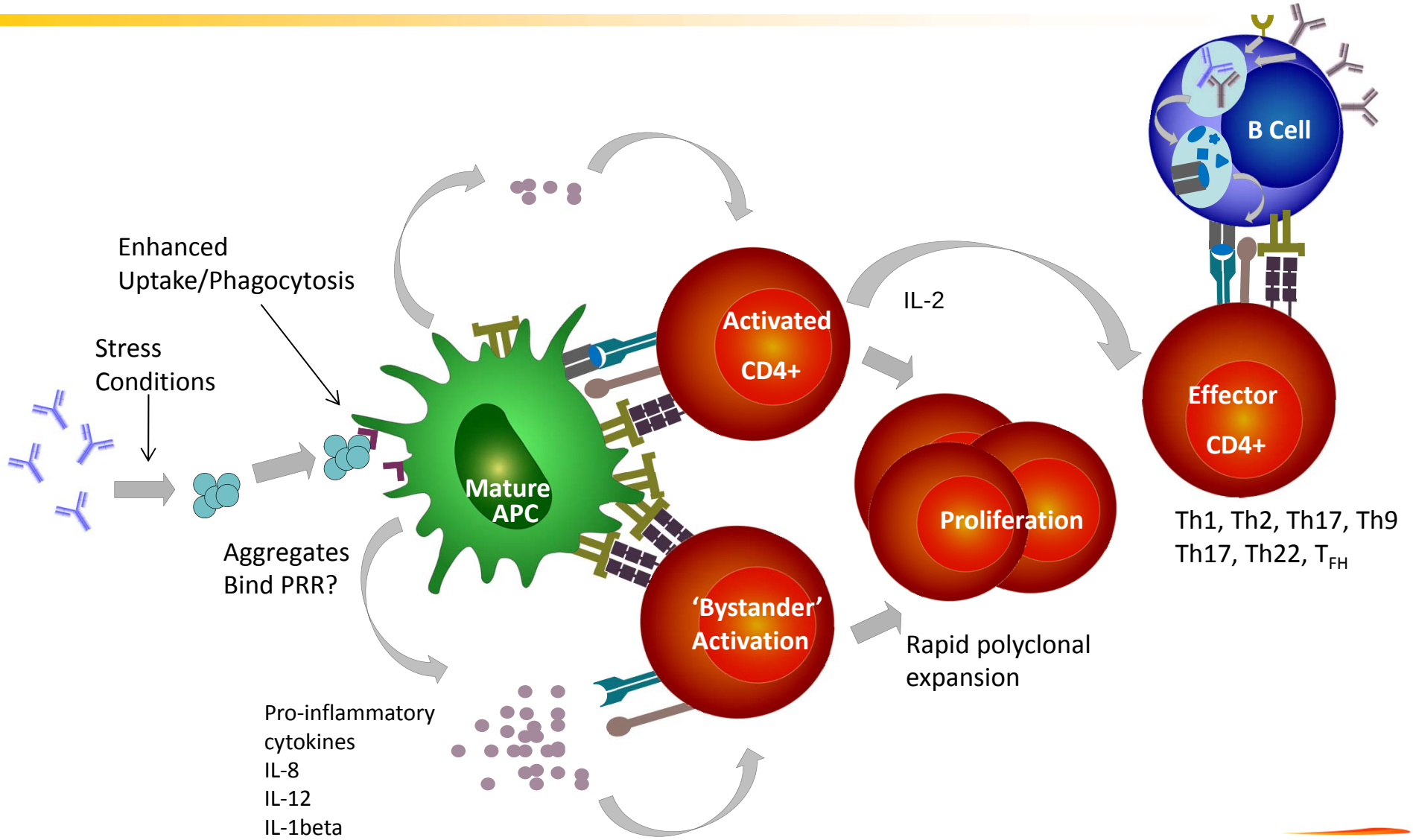




Cytokine Secretion Summary

- Shaking/stirred samples consistently show enhanced T cell activation – implications for transport, handling.
- Metal oxidised sample confirmed animal study results (van Beers 2011)
- Enhanced secretion of proinflammatory cytokines from DC.
- Preliminary Data suggests upregulation of CD80, CD86, CD40L.
- IL-10 upregulated in both PBMC and DC cultures. Suggesting activation of monocytes through PRR.
- DC increased IL-1B – mechanism for lymphocyte activation. Suggests a role for NLR rather than TLR?

Proposed Mechanism of Action



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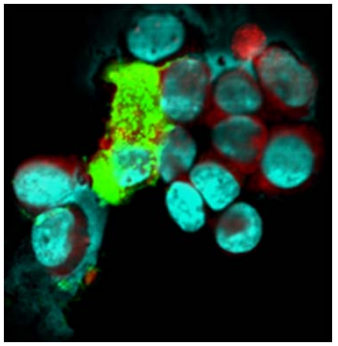
Conclusions

- Protein aggregates can enhance *in vitro* T cell stimulation.
- Enhanced stimulation influenced by aggregate e.g. size, quantity and/or physiochemical properties.
- Evidence of enhanced DC activation status. Enhanced uptake, potential multimeric interaction with PRR (TLR or NLR?), increased expression of co-stimulation molecules, proinflamatory cytokine secretion.
  - Hou et al 2008 – CpG multimers bind to TLR9 = enhanced DC maturations – T cell response.
- *Future directions-Tracking fluorescent labelled aggregate through APC, DC phenotyping, Caspase 1 inhibitor, TLR blocking antibodies.*

Contributors



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