



# Influence of Aggregates on *In Vitro* T Cell Responses

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Antitope

EIP Tuesday 7<sup>th</sup> February

# Aggregation of Protein Therapeutics

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- 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

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- 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° and/or 3° structure)
  - Small <1µm or large >10µm (typically hydrophobic)

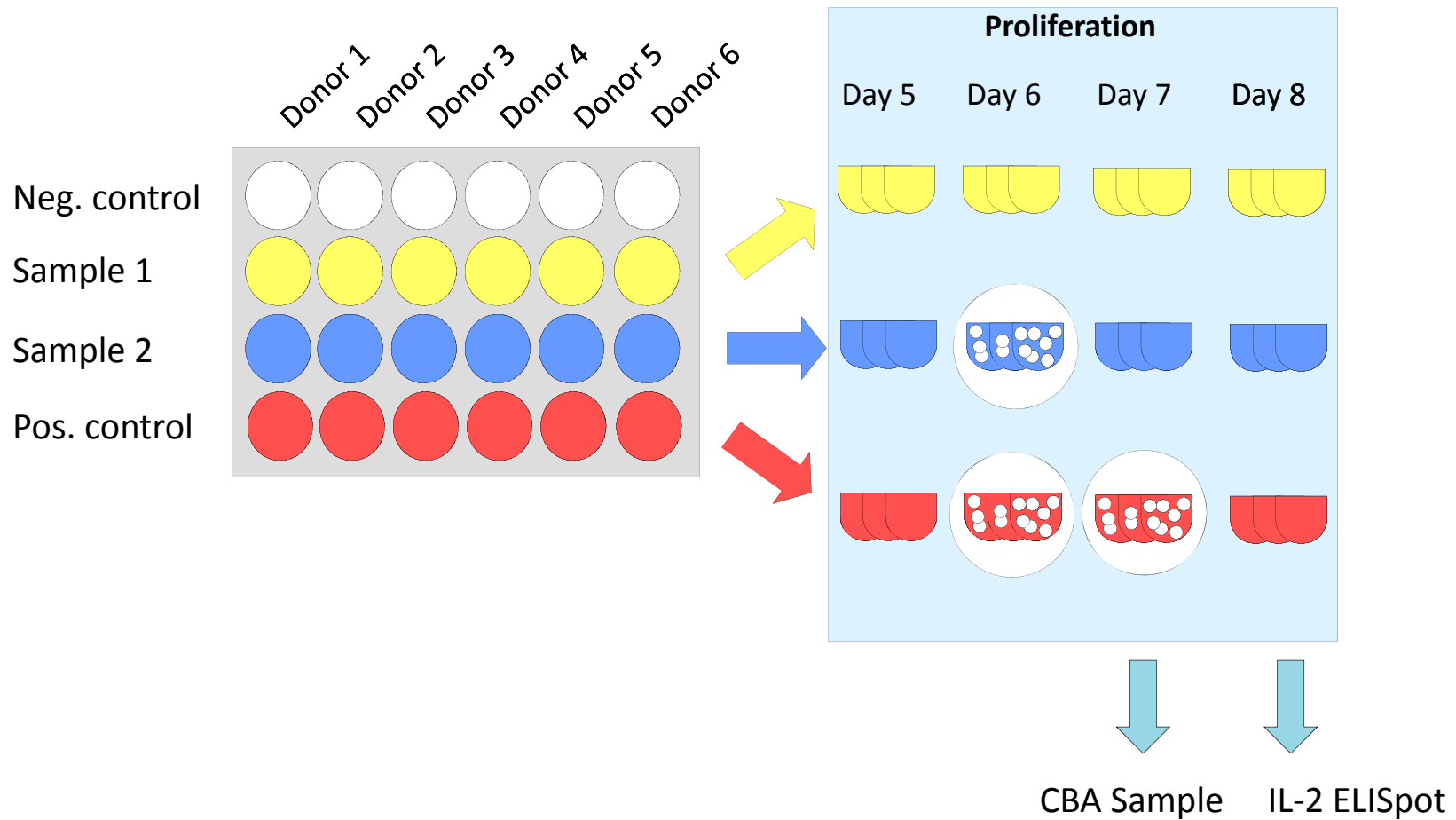
# Study 1:

## *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

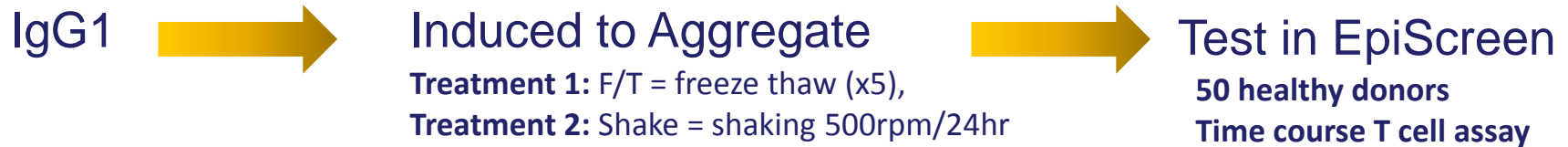


# Study 1:

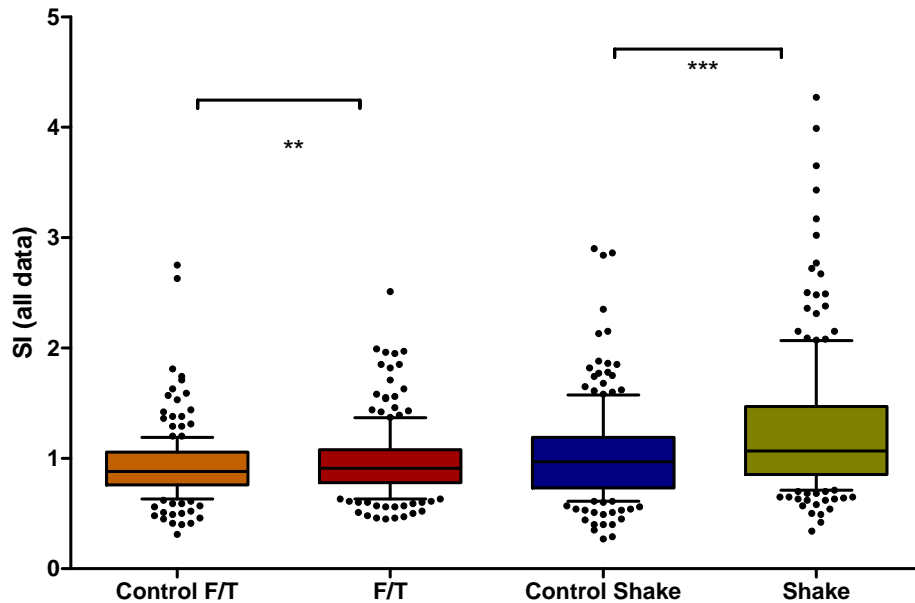
## Overview Generation of IgG1 Aggregates

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	<ul style="list-style-type: none"> <li>• non-covalent aggr.</li> <li>• intact secondary &amp; tertiary structure</li> </ul>	<p>&lt; 0.1% particles larger 1<math>\mu</math>m 0.2% larger soluble aggr.</p> <p>1.8% dimers 97.3% monomer</p>
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	<ul style="list-style-type: none"> <li>• mainly non-covalent aggr.</li> <li>• loss of secondary and tertiary structure</li> <li>• hydrophobic aggr.</li> </ul>	<p>14% large insoluble aggr.</p> <p>0.5% soluble aggr. 1.3% dimer/trimer 84% monomer</p>
Control Shake		Unstressed control	native IgG	1.5% dimers 98.0% monomer

# Study 1: In Vitro T Cell Stimulation with IgG1 Aggregates



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 $\geq$ 2.00,  $p < 0.05$  (cpm or spw test vs baseline)

	<b>Control Glu</b>	<b>Glu</b>	<b>Control Heat</b>	<b>Heat</b>
<b>Frequency</b>				
<b>Proliferation</b>	4%	16%	6%	12%
<b>IL-2</b>	4%	16%	8%	16%
<b>Both</b>	2%	16%	6%	12%

8 fold

2 fold

Insulin Study  $\rightarrow$  Glutaraldehyde Stress  
High frequency soluble aggregates

IgG Study  $\rightarrow$  Shaking Stress (IgG Study)  
High frequency large insoluble aggregates

Loss of  
tertiary  
structure

# Study 2:

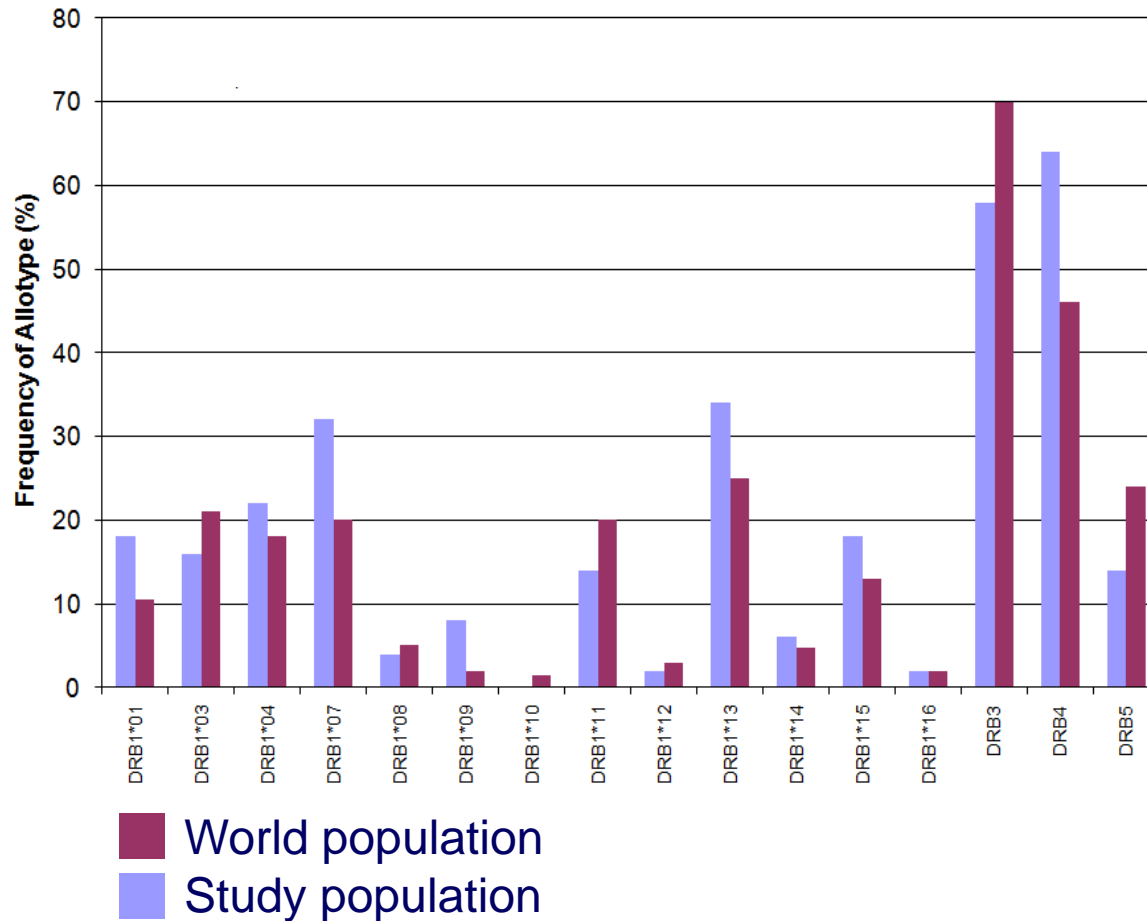
## Humira and Herceptin Aggregates

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- 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.

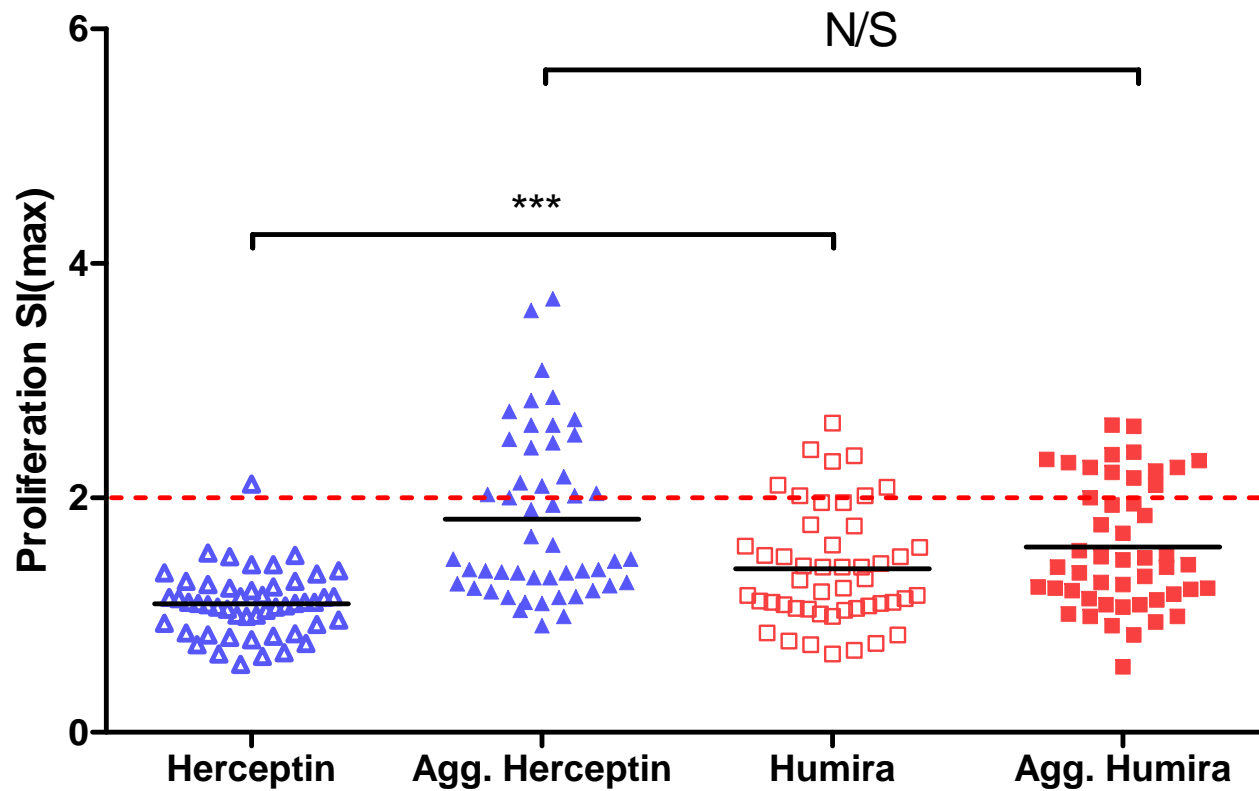
# Study 2: Donor Selection

50 healthy donors

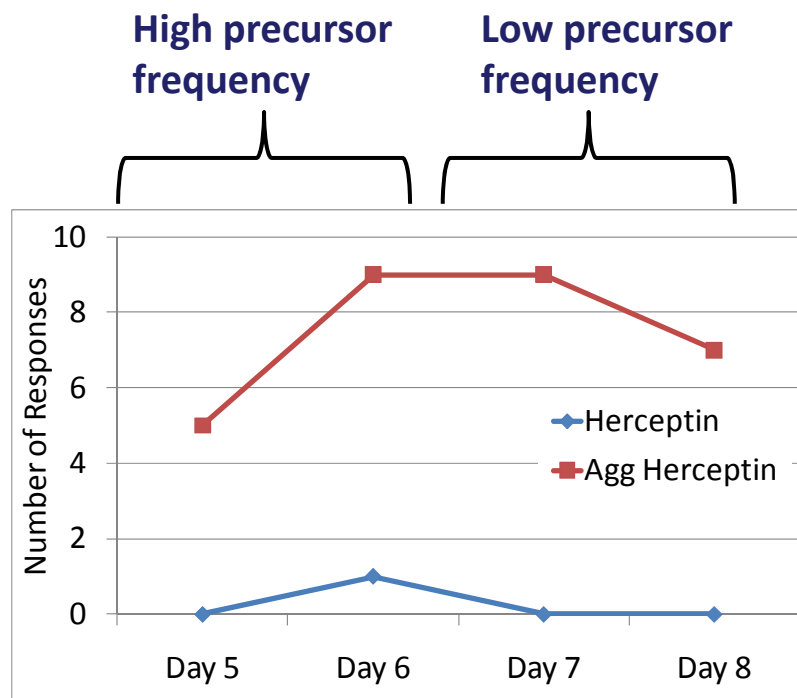




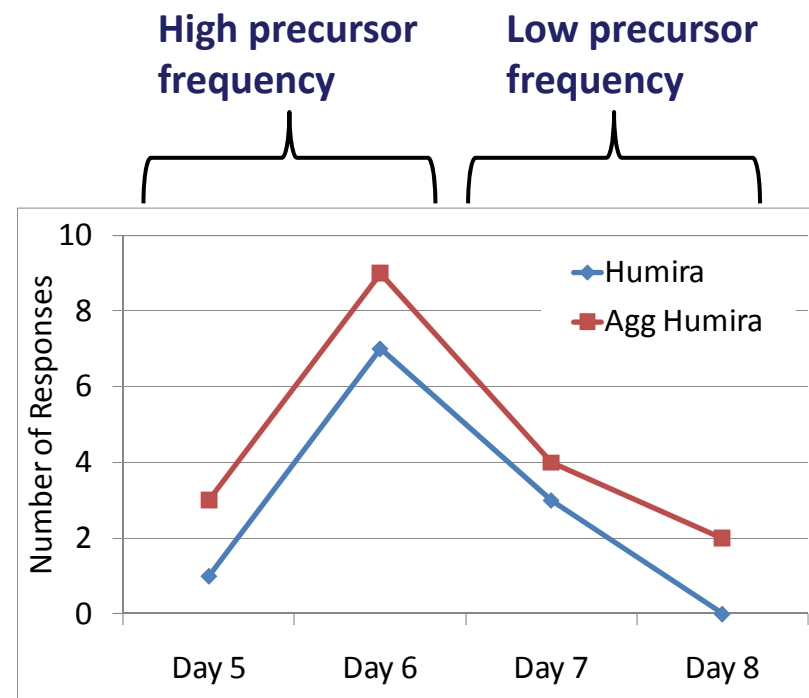
# Study 2: Aggregation Induces High Magnitude T Cell Responses



# Study2: T Cell Precursor Against Aggregated Antibody



Herceptin



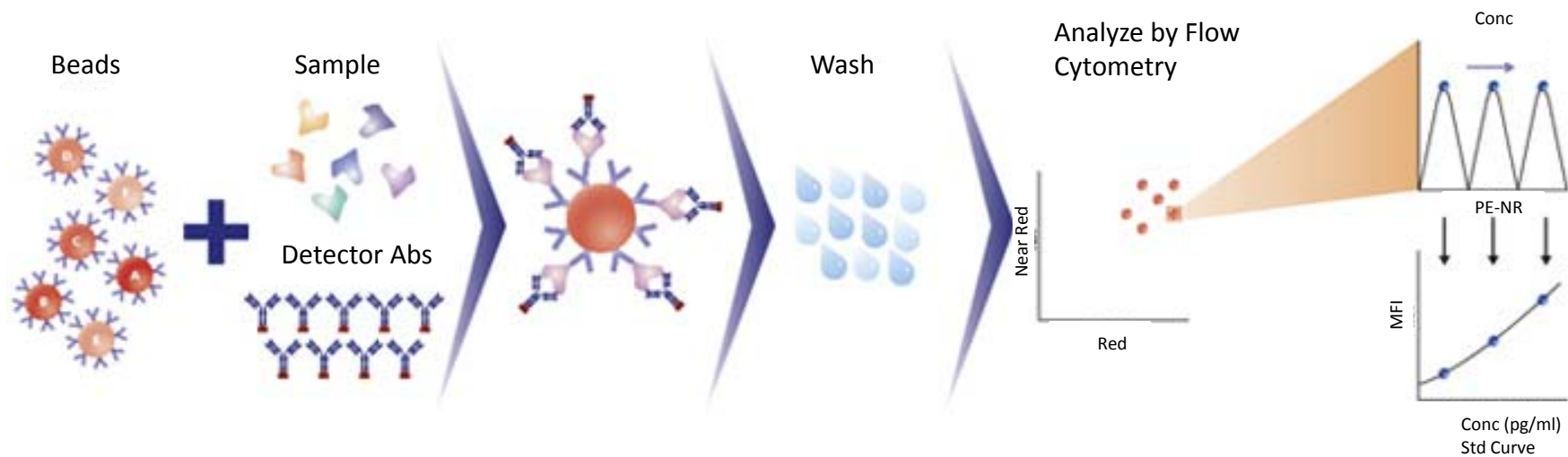
Humira

# Study 1 and 2: Conclusions

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- 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)



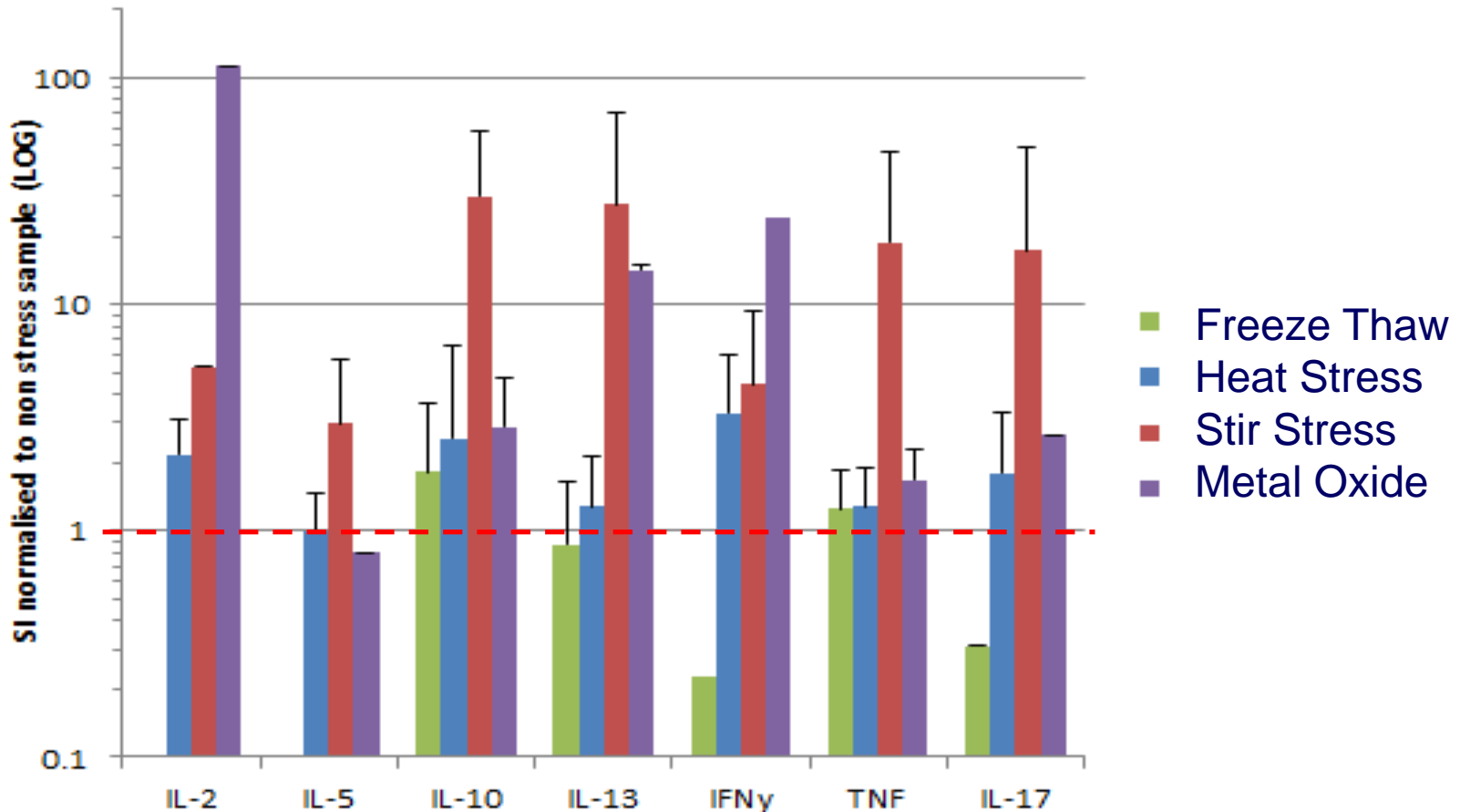
1. Capture beads have a unique fluorescence intensity and are coated with a capture antibody specific for a single cytokine.
2. A combination of different beads is mixed with a sample or standard + mixture of detection antibodies that are conjugated to a reporter molecule (PE).
3. Samples are acquired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

# Study 3: Cytokine Secretion from Aggregate Treated CD8<sup>+</sup> Depleted PBMC and DC

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- 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.

# Study 3, Cytokine Profile: Antibody Aggregate Stimulated CD8<sup>+</sup> Depleted PBMC



**Slide 17**

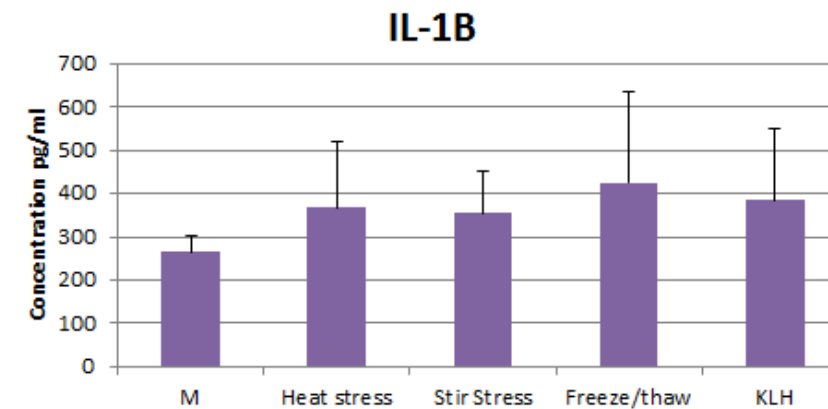
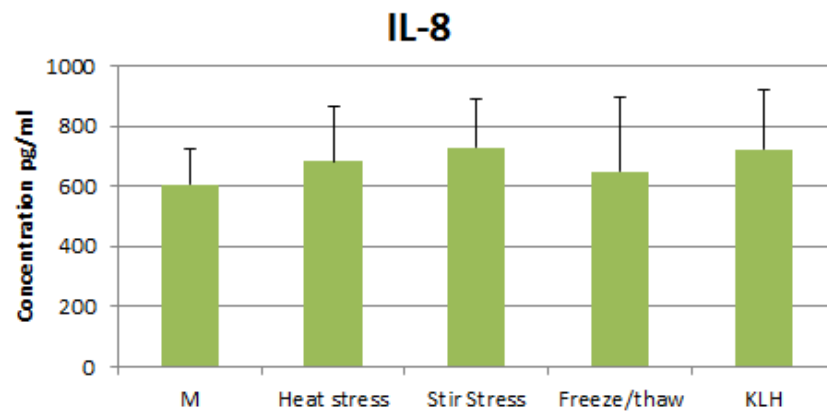
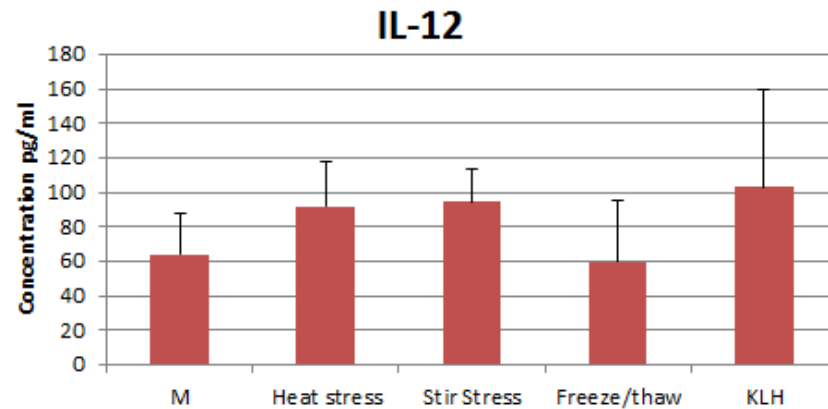
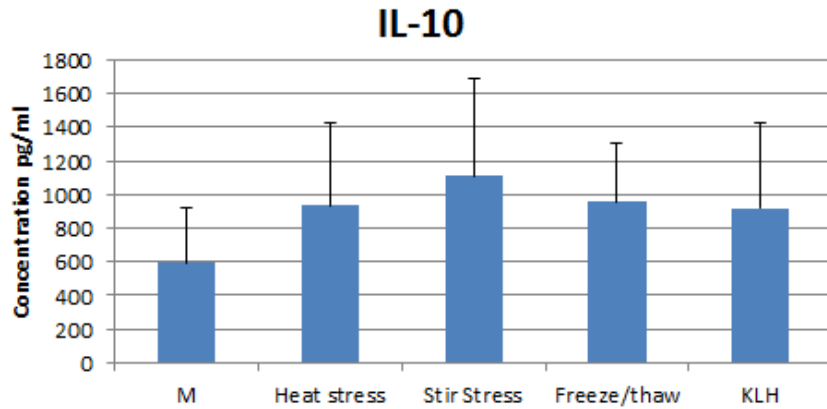
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**CB1**

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

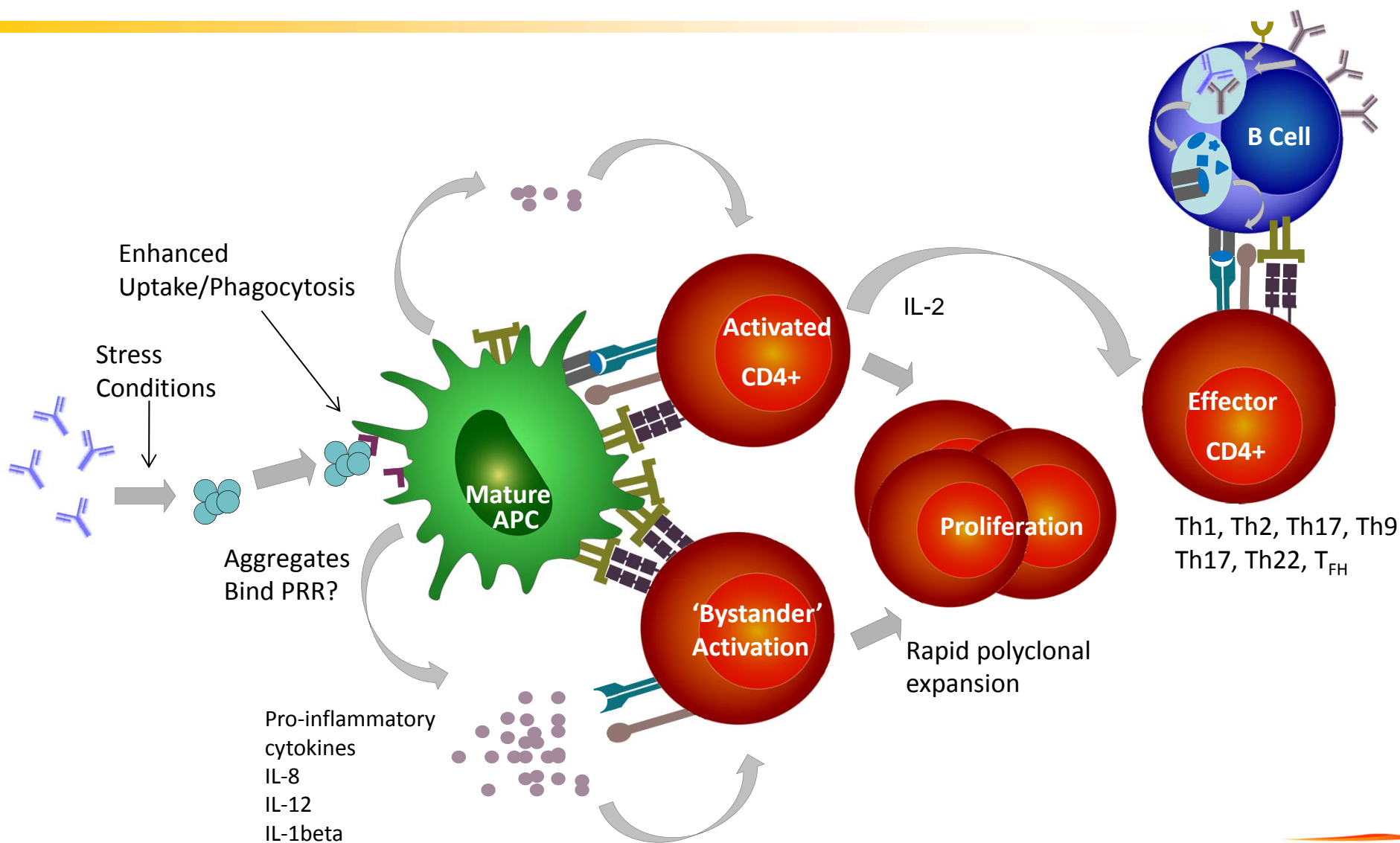


# Cytokine Secretion Summary

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- 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



# Conclusions

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- 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, proinflammatory 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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## **Immunology Group**

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