Testing Antibody Aggregates for Immunogenic Potential in Different Model Systems

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January 9, 2011
Elements of Immunogenicity

- Immunogenicity is ability of protein to induce an immune response usually measured as anti-drug antibodies (ADA)
- B cells, T cells, and antigen presenting cells play key role in immunogenicity
- Normally self reactive Ab expressing B cells and T cells are deleted in bone marrow/thymus or tolerized in periphery
- Antigen modifications may break tolerance to antigen leading to ADA usually in T-dependent manner
- T cells recognize a linear peptide sequence of processed antigen in context of MHC II molecules
- B cells recognize a conformation/structure and also process antigen
- Ag+ T cells help Ag+ B cells to expand and produce high affinity antibodies
### 3 types of Tools for Predictive Analysis of Immunogenicity Risk

<table>
<thead>
<tr>
<th>Role</th>
<th>Technique</th>
<th>Sequence</th>
<th>Protein Processing</th>
<th>Physical State</th>
<th>Dosing</th>
<th>Patient Immune Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>In silico (Tepitope)</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>In vitro</td>
<td>Y</td>
<td>Y</td>
<td>N/Y*</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>In vivo (mice)</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
</tbody>
</table>

* Dendritic cell assays
Engineering and Several Stability Evaluation Activities Facilitate Early Management of Potential CQAs at Amgen

**Very Early Stage/Early Stage candidates**

- **Sequence Analysis and Engineering Tier 1**
- **Limited Biophysical Stability**
- **Limited Formulation**
- **Immunogenicity Prediction**
- **Bioactivity Assessment**

**Tier 2**

- **Final Candidates**
- **Production Cell Line Development**
Preclinical Immunogenicity Assessment: Current Process in Molecule Assessment

1. **Candidate(s)**
   - **In silico Analysis**
     - **Prioritization**
       - **Low Risk**
       - **Higher Risk**
         - **In vitro Analysis**
           - **“De-immunization”**
             - **In silico Analysis**
               - **Development**
Example: Selection of Lead Candidates to Target ‘X’ Utilized Data From All Early MA Parameters

<table>
<thead>
<tr>
<th></th>
<th>Heavy Chain Isotype</th>
<th>pl</th>
<th>$K_D$</th>
<th>In-silico Predicted Non-Tolerant Epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First bin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mab1</td>
<td>VH1</td>
<td>7.4</td>
<td>11 pM</td>
<td>7</td>
</tr>
<tr>
<td>Mab2</td>
<td>VH1</td>
<td>7.6</td>
<td>14 pM</td>
<td>5</td>
</tr>
<tr>
<td>Mab3</td>
<td>VH1</td>
<td>7.8</td>
<td>24 pM</td>
<td>1</td>
</tr>
<tr>
<td>Mab4</td>
<td>VH4</td>
<td>7.4</td>
<td>13 pM</td>
<td>5</td>
</tr>
<tr>
<td><strong>Second bin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mab5</td>
<td>VH3</td>
<td>6.7</td>
<td>&lt;10 pM</td>
<td>2</td>
</tr>
<tr>
<td>Mab6</td>
<td>VH1</td>
<td>7.0</td>
<td>&lt;10 pM</td>
<td>3</td>
</tr>
<tr>
<td>Mab7</td>
<td>VH3</td>
<td>6.6</td>
<td>38 pM</td>
<td>3</td>
</tr>
<tr>
<td>Mab8</td>
<td>VH3</td>
<td>6.6</td>
<td>33 pM</td>
<td>2</td>
</tr>
</tbody>
</table>

Early MA identified Mab6 to be viscous at high concn. and hence eliminated; this Mab was also flagged for charged patch
But Sequence Analysis is Not Always Predictive of Immunogenicity Risk...

- In-silico computational tools
  - Help identify MHC class II binding linear peptide sequences
  - But cannot distinguish conformation and non-conformational epitopes (B cell epitopes?)
  - Overprediction of immunogenicity risk
  - Self-tolerance not accounted in tools
  - Do not consider factors beyond sequence that can break tolerance or enhance immunogenicity
    - Impurities
    - Aggregation
    - Route
    - Dose
    - Disease Indication
Goals of Amgen’s CQA Immunogenicity Team

– Test whether chemical and physical changes to protein therapeutics cause potential risk for immunogenicity
  • Develop human IgG tolerant in-vivo models to assess immunological impact
  • Evaluate in-vitro models for predicting potential risk of immunogenicity
– Create and characterize protein particles and aggregates for testing
– Can any clinical correlation with types of aggregates and immunogenicity be deduced
Once Validated and Well Characterized, These *In-vitro* or *In-vivo* Assays Envisioned to be Part of Final MA

- **Very Early Stage/Early Stage candidates**
  - 10-30 candidates

- **Sequence Analysis and Engineering Tier 1**
  - **Tier 2**

- **Biophysical Stability**
- **Formulation Assessment**
- **Immunogenicity Prediction**
- **Equal Bioactivity**

- **Final Candidates**
  - **Production Cell Line Development and Full MA**

- **In-silico**

In-vitro and in-vivo immunogenicity assays
Biopharmaceutical Aggregates can be Induced

<table>
<thead>
<tr>
<th>Steps During the Manufacturing Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation</td>
</tr>
<tr>
<td>Purification</td>
</tr>
<tr>
<td>Formulation</td>
</tr>
<tr>
<td>Storage</td>
</tr>
<tr>
<td>Shipping</td>
</tr>
<tr>
<td>Administration</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stress Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
</tr>
<tr>
<td>Freeze-thaw</td>
</tr>
<tr>
<td>Crosslinking</td>
</tr>
<tr>
<td>Protein concentration</td>
</tr>
<tr>
<td>Formulation change – pH, salt</td>
</tr>
<tr>
<td>Addition of extractables/leachables</td>
</tr>
<tr>
<td>Chemical modification</td>
</tr>
<tr>
<td>Mechanical Stress</td>
</tr>
<tr>
<td>Surface effects</td>
</tr>
<tr>
<td>Nano-particles</td>
</tr>
</tbody>
</table>
Protein Aggregates have Widely Varying Properties

Aggregate types

- Distribution of particle sizes
- Shape
- Reversibility/ Stability
- % Aggregation
- Total amount of Aggregate
- Native vs non-native conformation
- Regular array/higher order structure
- Covalently bound molecules
- Chemical Modification
- Soluble vs. pellet fraction
- Density
- Exposed T and B-cell epitopes
- Covalent vs. non-covalent bonds
- Crystalline structure
# mAb1 Aggregates were Generated under a Variety of Conditions

**mAb1 - IgG<sub>2</sub> monoclonal antibody**

<table>
<thead>
<tr>
<th>Stress Treatment</th>
<th>Sample Name</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) No treatment</td>
<td>untreated</td>
<td>-</td>
</tr>
<tr>
<td>(2) Freeze-thaw</td>
<td>ft-slow</td>
<td>-80°C/37°C (10 cycles)</td>
</tr>
<tr>
<td></td>
<td>ft-fast</td>
<td>Liquid nitrogen/37°C (10 cycles)</td>
</tr>
<tr>
<td>(3) Storage</td>
<td>store</td>
<td>37°C, 2 months</td>
</tr>
<tr>
<td>(4) pH change</td>
<td>pH 3.5</td>
<td>10mM Acetate pH 3.5, 4.3, 8.5, or 11, 37°C for 16hr</td>
</tr>
<tr>
<td></td>
<td>pH 4.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 8.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 11</td>
<td></td>
</tr>
<tr>
<td>(5) Crosslink</td>
<td>xlink</td>
<td>EDC/NHS, quench with hydroxylamine</td>
</tr>
<tr>
<td>(6) Mechanical</td>
<td>pipette</td>
<td>Pipette 100x</td>
</tr>
<tr>
<td></td>
<td>agitate-4C-so</td>
<td>Agitate at 4°C, 7d, 500 µl in 3cc vial, with and without silicon oil</td>
</tr>
<tr>
<td></td>
<td>agitate-4C-so⁺</td>
<td></td>
</tr>
<tr>
<td></td>
<td>agitate-so⁻</td>
<td>Agitate at 22°C, 3d, 500 µl in 3cc vial, with and without silicon oil</td>
</tr>
<tr>
<td></td>
<td>agitate-so⁺</td>
<td></td>
</tr>
<tr>
<td></td>
<td>syringe-so⁻</td>
<td>Push through a Daikyo Crystal Zenith syringe (no silicon oil) 50x</td>
</tr>
<tr>
<td></td>
<td>syringe-so⁺</td>
<td>Push through a disposable syringe (contains silicon oil) 50x</td>
</tr>
<tr>
<td></td>
<td>stir-20h</td>
<td>Stir for 1-3 days, 2ml in 5cc w/ teflon stirrer bar</td>
</tr>
<tr>
<td></td>
<td>stir-3d</td>
<td></td>
</tr>
<tr>
<td>(7) Hydrogen peroxide oxidation</td>
<td>H₂O₂</td>
<td>H₂O₂, 37°C for 16hr, quench with Methionine, dialyze overnight</td>
</tr>
<tr>
<td>(8) Metal catalyzed oxidation</td>
<td>metal</td>
<td>CuSO₄ and ascorbic acid, 37°C for 16hr, chelate with EDTA, dialyze overnight</td>
</tr>
<tr>
<td>(9) Heat</td>
<td>65C/pH 8.5</td>
<td>65°C, 10 mM Acetate pH 8.5 for 1hr</td>
</tr>
<tr>
<td></td>
<td>90C</td>
<td>90°C for 16 hr</td>
</tr>
</tbody>
</table>

All aggregates were made at 1-10 mg/ml in 10 mM Acetate pH 5 except where a pH change is noted
Panel of Particle Images Showing Distinct Morphological Features

Images taken by MFI: 1 mg/ml mAb1 aggregates diluted 20-220X in 10 mM Acetate pH 5, 0.5 ml analyzed.
## Aggregate Characteristics

<table>
<thead>
<tr>
<th>Stress Treatment</th>
<th>Particle Conc. ≤1µm</th>
<th>Particle Conc. 2-10 µm</th>
<th>Degree of Native Conformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>++</td>
<td>+</td>
<td>native</td>
</tr>
<tr>
<td>syringe-so⁺</td>
<td>++++</td>
<td>+++</td>
<td>partially native</td>
</tr>
<tr>
<td>stir-20h</td>
<td>++++</td>
<td>++++</td>
<td>partially native</td>
</tr>
<tr>
<td>stir-3d</td>
<td>++</td>
<td>+++</td>
<td>mostly unfolded</td>
</tr>
<tr>
<td>65C/pH 8.5</td>
<td>+++</td>
<td>+++</td>
<td>mostly unfolded</td>
</tr>
</tbody>
</table>

- **mAb1 aggregates**
In Vitro Assays To Assess Immunogenicity Risk of Human Ig Aggregates

- **Applications**
  - Confirm in-silico prediction for top candidates
  - **Utilize as in-vitro human model for differentiating monomers from other process mediated issues**

- **Outcome**
  - processing and presentation potential of a protein therapeutic
  - Determine role of target mediated immunogenicity (for immune-based targets)
  - Should cover a broad range of clinically relevant MHC II alleles, T cell repertoire and/or B cell repertoire and response levels

- **Types of assays**
  - Dendritic cell and PBMC based assays
  - T cell responses currently being tested
Dendritic Cell Assays

- Dendritic cells are the primary antigen presenting cells
- Goal is to develop methodologies for assessment of immunogenicity of aggregates, formulations, immunomodulation

DC-Ag Interaction Cascade

**Activation:**
- ↑ MHC, LFA, ICAM, CD80
- Cytokine release

**Ag Binding:**
- ↑ TLR 7/9,
- P-Y such as BTK2

**Presentation:**
- T-cell activation,
- Cytokine release

**Processing:**
- Ubiquitination,
- Cellular elongation

Each stage of this cascade can be assessed by the event specific markers
Dendritic Cell Assay Informs on First Stage of Immune Response: 1) Processing of Protein and 2) Activation of Cell

- PBMC purified monocytes
- Differentiated DC
- IgG2 control
- Mab1

1. untreated IgG in A5 buffer
2. Syringe SO- IgG
3. Syringe SO+ IgG
4. Stirring 1 day IgG
5. Stirring 3 day IgG
6. 65c pH8.5 treated IgG
7. 80c IgG
8. A5 buffer
9. A5+syringe SO(+)

MCP-1 chemokine expression in imDCs

Graph showing RU (resonance units) levels for different treatments.
Peripheral Blood Mononuclear Cells (PBMCs) Based In-Vitro Assays

• Objectives
• Assess the impact of aggregated or monomeric forms of fully human monoclonal antibody based therapeutics on naïve human peripheral blood mononuclear cells (PBMC) for:
  – Early innate phase of the immune response to evaluate the role of aggregates in induction of inflammatory test site reactions
  – Late adaptive immune response of PBMC-derived T-cells to evaluate the development of memory response leading to neutralizing antibody against the therapeutic
Monocyte Derived Cytokines Released in Response to Certain MAb1 Aggregates

Stimulation Index = mean cytokine concentration (pg/ml) of PBMCs in response to aggregates over untreated for responding donors
Bar height indicates the average stimulation index of responding donors
GM-CSF, IL-4, IL-12p40, IL-12p70, and IL-1ra were tested in all 22 donors and did not show a significant increase.
Untreated showed an increase in cytokine secretion over media for certain cytokines
## Correlation of Aggregate Characteristics and the Induction of the Innate Immune Response

<table>
<thead>
<tr>
<th>Stress Treatment</th>
<th>Particle Conc. ≤1µm</th>
<th>Particle Conc. 2-10 µm</th>
<th>Degree of Native Conformation</th>
<th>IL-1β No. Res. Donors (%)</th>
<th>IL-6 No. Res. Donors (%)</th>
<th>MCP-1 No. Res. Donors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>++</td>
<td>+</td>
<td>native</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>syringe-so+</td>
<td>++++</td>
<td>+++</td>
<td>partially native</td>
<td>14</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>stir-20h</td>
<td>++++</td>
<td>++++</td>
<td>partially native</td>
<td>29</td>
<td>29</td>
<td>38</td>
</tr>
<tr>
<td>stir-3d</td>
<td>++</td>
<td>+++</td>
<td>mostly unfolded</td>
<td>14</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>65C/pH 8.5</td>
<td>+++</td>
<td>+++</td>
<td>mostly unfolded</td>
<td>10</td>
<td>14</td>
<td>24</td>
</tr>
</tbody>
</table>
### Summary of Cytokines that are Secreted in PBMC or DC Cultures in Response to Aggregates

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Reported Cell Source</th>
<th>Reported Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α, IL-1β</td>
<td>Monocytes, macrophages, NK cells, neutrophils</td>
<td>Requirement in NF-κB activation due to IC induced injury, stimulates T-B cell maturation</td>
</tr>
<tr>
<td>IL-6</td>
<td>Monocyte, Macrophages, FDC</td>
<td>Innate response to PAMP Germinal centre reaction, IgG responses</td>
</tr>
<tr>
<td>IL-8</td>
<td>Macrophages, endothelial cells</td>
<td>Chemotaxis in neutrophils, involved in innate immune response</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocytes, Osteoclasts, activated T cells</td>
<td>Recruits monocytes, memory T cells and dendritic cells to the site of inflammation and injury</td>
</tr>
<tr>
<td>MIP-1α, MIP-1β</td>
<td>Monocytes, macrophages</td>
<td>Leukocyte chemotaxis, attracts neutrophils, basophils, and T-cells</td>
</tr>
<tr>
<td>TNF-α</td>
<td>T cells, macrophages</td>
<td>Systemic inflammation and regulation of immune cells</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Monocytes</td>
<td>Cell proliferation, migration, tissue remodeling</td>
</tr>
</tbody>
</table>
Summary of In-Vitro Assays

- Some protein aggregates can induce a pro-inflammatory cytokine response profile in PBMCs and Dendritic cell assays by stimulating APCs
  - APCs release IL-10, IL-1β, IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, and TNF-α in response to protein aggregates
  - These cytokines play an important role in chemokine mediated cell migration, as well as stimulation of T cell and B cell proliferation
  - Aggregates created by stirring, mAb1 (stir-20h) and mAb2 (stir-20h and stir-3d), induced the highest levels of cytokine release both by magnitude and number of responders
  - Protein aggregates with the highest particle counts and partially native-like conformation had the greatest number of responders
What are the *in vivo* Model Options for Immunogenicity Testing of Aggregates

- Non-human, Non-transgenic animals
  - Non-human primates, mice, rats, rabbit etc
  - Non-tolerated to human Ig
  - Recognize human antibodies as foreign and can mount humoral immune responses to them
  - Might be unable to distinguish non-aggregated and aggregated human antibody

- Knock-out model
  - Used for assessment of immunogenicity to human antigens genetically deficiency in some patients
  - Human factor VIII (Delignat S et al. Hematologica, 2007)
    - Exogenous FVIII induces 15-30% inhibitory antibodies
    - Knock-out mouse model for evaluating the effect of both non-aggregated and aggregated FVIII on immunogenicity
Current *in vivo* Models in Immunogenicity Testing of Protein Aggregates

- **Transgenic models: immune tolerant for test protein**
  - Human IFNα2 (*Hermeling S et al, J Pharm Sci, 2006*)
    - Tg model for studying potential factors affecting the high immunogenicity in clinic (20%)
    - Aggregated protein, dose and injection route contribute to immune response
  - Human IFN-β (*Hermeling S, Pharm Res, 2006*)
    - Tg model to study immunogenicity of three products
    - Betaseron/Betaferon (rhIFNβ-1b, E. coli), Avonex (rhIFNβ-1a, CHO) and Rebif (rhIFNβ-1a, CHO)
    - Betaseron breaks immune tolerance of Tg mouse but not rhIFNβ-1a
  - Hybrid transgenic immune tolerant mouse model for huIFN-beta (Beers et al, 2010)
    - Reported lack of memory antibody responses to bulk, reformulated and pH stressed rhIFN-beta samples
  - Human Insulin (*Otteson JL et al, Diabetologia, 1994*)

- **To date, in-vivo models accounting for self-IgG tolerance have not been established/studied by others for immunogenic potential of human IgG aggregates**
Amgen XenoMouse® Technology

- Transgenic human IgG mouse
- A robust tool for generating therapeutic human antibodies

Is XenoMouse a potent mouse model for studying immunogenicity of antibody drugs?
XenoMouse®: Primary Tool for Generating Fully Human IgG Therapeutics

Only human IgG but no mouse IgG is produced.
Phenotype of XenoMouse® Closely Matches Human B Cell Development and Ig Expression

• Human immunoglobulin (Ig) transgenic mouse
  – Murine IgH and IgLκ loci were functionally inactivated
  – Human IgH and IgLκ/λ genes were introduced through YAC
• Diversified VH and VL and cis-regulatory elements retain V repertoires of human B cells
• Like human, XenoMouse sustains expression of human IgM/IgD on B cells representing mature naïve human B cell population
• Like human, XenoMouse undergoes Ig class switching to IgG, somatic mutation and affinity maturation

Human Ig of the same isotype or same IgG subclass is recognized as self-protein in XenoMouse®
Can XenoMouse be Used to Predict the Immunogenicity of Therapeutic mAbs?

- We believe XenoMouse animals should be tolerant to human Abs with a wide array of variable genes
- They represent a human-like v-repertoire of responses
- Conceptually similar to transgenic mice (Tg) expressing IFNα2a/2b, IFN-β, human insulin

- Although responses to antigen in the presence of adjuvant are sufficient, they are not robust enough to test the role of aggregates in formulated protein samples
  - Mismatched BCR: Human Ig but mouse signaling components
Xeno Heterozygous Mouse as a Model System for Tolerance

- Cross-breed with wild type mouse
- Retain self-tolerant to human IgG
- Restore functional murine immune system
Expected Characteristics of Xeno/BL6 Heterozygous Mouse

• Murine IgH and IgL loci are functionally re-activated
• Human IgM/IgD (huBCR) and IgG are expressed
• Diversified V repertoires of mouse B cells gained
• Should undergo both murine and human Ig class switching, somatic mutation and affinity maturation
• Recognize foreign antigens and induce murine and human humoral responses

Murine Ig and human Ig are recognized as self-protein thus accounting for tolerance factor to human IgG
Expected Lymphocyte Maturation in Xeno/BL6 Heterozygous Mouse

- Mature murine T cells
  - Murine MHC restricted and self-antigen (including human IgG) tolerant
- Mature murine B cells
  - Both murine-Ig containing BCR carried B cells and human –Ig containing BCR carried B cells exist
  - Murine BCR carried B cells will be dominant to human BCR carried B cells and both murine and human IgG are tolerated

Expect it to be an immune competent model for human IgG immunogenicity study
Hypothesis of Immune Responses in Xeno Heterozygous Mouse

Ag processing & presentation

Presenting to Th cells

Ag-specific B cell activation

Activation

Bu-Ig BCR

Murine anti-hulG Abs (High)

Human anti-hulG Abs (Lower)
Characterization of Xeno Heterozygous Mouse: Human and Murine BCR-expression

Both Xeno/B6 and Xeno/129 het mice gain normal B220+ B cells after crossing to wt mouse;
Only limited B cells express hulg(M+G) on the surface:
98% of B cells express mulg(M+G); 1-3% for hulgM, and 0.1-0.3% for hulgG.
Summary of Earlier Work on Optimization of \textit{In Vivo} Model

- \textit{1st In vivo} study in wt mouse for determination of dose/injection frequency (June, 2009)
  - SC route
  - Immunization regimen\#1 (low dose, more injections) induces stronger antibody response than regimen\#2 (high dose, less injections)
  - Metal catalyzed aggregates induced sustained antibody response

\textit{B6/129} wt mice
(No tolerance to hulgG2/kl)
In vivo Study for Addressing Immune Responses Against Modified Drug in Xeno Het Mouse Model

Modification

- Monomer
- hulgG2-coated Microspheres
- hulgG2 1-d stirring
- hulgG2-TCE-KLH & Adjuvant

Immunization

- Xeno/B6 het mouse (10-12 mice/group)
  - Low dose, 7x immu. in 3 wks

Characterization

- Sacrifice 2 mice weekly at early time points
- Harvest SPC and BMC
- T/B cell activation studies
- Titration of anti-hulgG2 Ab (mulgG) weekly
Immunization and Sampling Scheme for Xeno-het Study

Sacrifice 2 mice for SPC & BMC Harvesting (in vitro T/B cell activation study)

Antigen Injection

Bleed animals weekly for preparing antisera
**Anti-drug Serum Titration to huIgG2 Samples**
*(S/N Ratio of Serum Titer Post/Pre)*

<table>
<thead>
<tr>
<th></th>
<th>2 wks</th>
<th>4 wks</th>
<th>8 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
<td><img src="image3.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

- Xeno-het tolerant to huIgG (huIgG2/kappa)
- Immune-tolerance can be broken by inclusion of Th epitopes
- Significant drug-specific antibody responses from huIgG2-TCE-KLH group were detected at 2 wks and remain high at end of study (8 wks)
## Anti-drug B-Cell ELISPOT in Xeno-het Mice Immunized with Modified huIgG2 Samples

### Significant drug-specific B cell responses in huIgG2-TCE-KLH and modified huIgG2 groups compared to hlIgG2 monomer
Summary

• The current study examined the effect of varying doses and route of immunization on anti-drug responses and found low dose, more frequent immunizations induce stronger immune responses

• Xeno-het mice expressed hu and mu Ig heavy and light chains and secrete both murine and human IgG and IgM

• Xeno-het mice showed self tolerance to huIgG2 sample; conjugation of huIgG2 molecule to the T-cell epitope and KLH breaks tolerance to the molecule

• All animals elicited anti-KLH responses demonstrating ability to respond to foreign antigen

• Human IgG tolerant animal model has been established and is practical for further studies of aggregates and chemical modifications related immunogenicity concerns
Unanswered Questions That Will be Addressed Next

- Impact of ordered arrays on anti-drug antibody responses
- Is oxidation alone sufficient to trigger immune response?
- Effect of agitation (as mimicked by stir conditions) on B and t cell responses
- Are these ADA responses transient or persistent?
Immunization Course in Xeno Het Mice for 3rd Study

Sacrifice 2 mice for SPC & BMC Harvesting
(in vitro T/B cell activation study)

wk
0 1 2 3 4 5 6 7 8

Antigen Injection

Bleed animals for preparing antisera

Rules to follow:

• Maintain animals in original same cage to avoid fighting, dermatitis and de-identification
• Randomized gender among study groups based on other immunization study at hybridoma lab
• Age range: 8-12 week old
Schematics of New *in vivo* Immunogenicity Study

- Animal Breeding (ensuring age, health check etc)
- Animal Lab
- Record / Group / Labeling
- Immunization
- Bleeding (sera collection and dilutions)
- ADA Measurement
- Sample Prep. (aggregate preparation, injection sample preparations)
- QC Study of Samples
- Tissue Harvesting: BM, Spleen, bleeding
- B-ELISPOT (anti-drug specific)
- T- Response
- Immunization
- Bleeding (sera collection and dilutions)
- ADA Measurement
- Immunization
- Bleeding (sera collection and dilutions)
- ADA Measurement
- Immunization
- Bleeding (sera collection and dilutions)
- ADA Measurement
Progression Stages of In-Vivo Immunogenicity Studies

In vivo Response of Aggregated hIgG

Possible Attritions to Immunogenicity

Correlation of Immunogenicity to Degree of Aggregation

More Studies of Potential Attributor(s)

- Dose & Route of Immunization
- Expand #animal In Study Group
- Apply to Other Therapeutic Abs
Other Future Directions

- Test impact of chemical modifications on immune response in in-vivo and in-vitro models
- Further validate in vitro human model systems
  - Quantitative evaluation of cytokines, and association of particle size and nature of aggregates on the activation of innate (T cell independent) and adaptive (T cell dependent) phases of the immune response
- Optimize proteomics for characterizing immune response profile in aggregate stimulated systems
Acknowledgements

- Toni Mire-Sluis
- Terry Goletz
- Naren Chirmule
- Jilin Sun
- Amy Batista
- Dan Mytych
- Martha Hokom
- Steve Swanson
- Kyla Gordon
- Karen Richmond
- Oscar Pan
- Aaron Winters
- Alberto Deloera
- Evelyn Pryor
- Denise Castañeda
- GEM Service

- Vivian Bi
- Margaret Karow
- Linda Narhi
- Marisa Joubert
- Arunan Kaliyaperumal
- Vibha Jawa
- Bruce Kerwin
- Catherine Eakin
- Allison Wallace
- Jette Wypych
- Quanzhou Luo
- Rupa Padaki
- Mark Michaels
Backups
Types of Protein Aggregates Reported to Elicit an Immune Response

Rigidly organized protein arrays in the micron range may be highly immunogenic
VSV-G and VLP and regularly spaced acrylamide polymers (5-10 nM) are immunogenic
  Dintzis et al., PNAS, 73 (1976) 3671-5.

Immune response of protein coated nanobeads and preferential internalization of protein coated aluminum adjuvants by DCs
  Morefield et al., Vaccine, 23 (2005) 1588-95.

Reports of protein aggregate immunogenicity in vivo give conflicting results
  Aggregates of IFN-γ: metal-catalyzed and pH/50°C induced aggregates (but not untreated, crosslinked, hydrogen peroxide or boiled) can break tolerance in transgenic mice.

Aggregates of FVIII: heat induced aggregates were less immunogenic than the monomeric protein.

Aggregates of rhGH: freeze-thaw and agitation induced aggregates were not able to break the tolerance of transgenic mice (freeze-thaw showed an enhanced response in wild-type mice)

no in vivo data on therapeutic immunoglobulin (IgG) aggregates available
# Classification Scheme for mAb1 Aggregates Based on Biophysical Characterization

<table>
<thead>
<tr>
<th>Class</th>
<th>Description</th>
<th>Stress Treatment</th>
<th>Particle Conc. ≤1 µm</th>
<th>Particle Conc. ≥2 µm</th>
<th>Particle Size (µm)</th>
<th>FTIR CC sup/pellet</th>
<th>Shift in λ max (nm)</th>
<th>% ANS Binding</th>
<th>Rev. (R) Part Rev. (PR) Irrev. (I)</th>
<th>Chem. Modified</th>
<th>% Agg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1</td>
<td>not aggregated</td>
<td>untreated, ft-slow, ft-fast, store, pH 3.5 to 8.5, pipette, agitate-4C-so⁻, agitate-so⁺</td>
<td>++</td>
<td>+</td>
<td>&lt;10</td>
<td>&gt;89/-</td>
<td>≤1</td>
<td>&lt;11%</td>
<td>-</td>
<td>&lt;+</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Class 2</td>
<td>metal containing</td>
<td>H₂O₂</td>
<td>++</td>
<td>+</td>
<td>&lt;5</td>
<td>99/-</td>
<td>1</td>
<td>11%</td>
<td>PR</td>
<td>++</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 11</td>
<td>++</td>
<td>++</td>
<td>&lt;20</td>
<td>92/71</td>
<td>1</td>
<td>9%</td>
<td>R</td>
<td>-</td>
<td>4%</td>
</tr>
<tr>
<td>Class 3</td>
<td>small, native, partially reversible</td>
<td>syringe-so⁻</td>
<td>-</td>
<td>+++</td>
<td>&lt;40</td>
<td>92/-</td>
<td>1</td>
<td>16%</td>
<td>PR</td>
<td>+</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>syringe-so⁺</td>
<td>++++</td>
<td>+++</td>
<td>&lt;50</td>
<td>96/32</td>
<td>1</td>
<td>31%</td>
<td>PR</td>
<td>+</td>
<td>10%</td>
</tr>
<tr>
<td>Class 4</td>
<td>small, native, irreversible</td>
<td>xlink</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>89/-</td>
<td>1</td>
<td>8%</td>
<td>-</td>
<td>-</td>
<td>3%</td>
</tr>
<tr>
<td>Class 5</td>
<td>small, partially native, partially reversible</td>
<td>stir-20h</td>
<td>++++</td>
<td>++++</td>
<td>&lt;20</td>
<td>98/42</td>
<td>1</td>
<td>14%</td>
<td>R</td>
<td>+</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>agitate-so⁺</td>
<td>++++</td>
<td>+++</td>
<td>&lt;5</td>
<td>89/50</td>
<td>1</td>
<td>52%</td>
<td>-</td>
<td>-</td>
<td>75%</td>
</tr>
<tr>
<td>Class 6</td>
<td>medium, mostly unfolded, partially reversible</td>
<td>stir-3d</td>
<td>++</td>
<td>+++</td>
<td>&lt;60</td>
<td>89/20</td>
<td>2</td>
<td>49%</td>
<td>R</td>
<td>++</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>65C/pH 8.5</td>
<td>++++</td>
<td>+++</td>
<td>&lt;130</td>
<td>100/26</td>
<td>4</td>
<td>95%</td>
<td>PR</td>
<td>++</td>
<td>85%</td>
</tr>
<tr>
<td>Class 7</td>
<td>large, unfolded, irreversible</td>
<td>90C</td>
<td>++</td>
<td>+++</td>
<td>&lt;220</td>
<td>42/19</td>
<td>4</td>
<td>100%</td>
<td>I</td>
<td>+++</td>
<td>95%</td>
</tr>
</tbody>
</table>