Fit-for-purpose validation of an *in vitro* immunogenicity risk assessment assay

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BioMedicine Design
Mitigation of biotherapeutic immunogenicity is needed to ensure benefit to patients, maintain commercial value and reduce attrition.

Multiple factors influence Immunogenicity:

- **Product**
  - Target
  - Mechanism of action
  - Sequence Structure
  - Post-translational modifications
  - Aggregation
  - Impurities
  - Formulation

- **Treatment**
  - Dose
  - Frequency
  - Duration
  - Route

- **Patient**
  - Disease
  - Medication
  - Genetic background
  - Co-medication

Mitigation by design
*In silico* and *in vitro* assays guide molecular design by assessing risks at key steps in the immune cascade, which can lead to ADA development.

1. APC activation
   - Therapeutic protein
   - *in silico* assays:
     - HLA binding prediction
     - Peptide presentation

2. Antigen Processing and presentation
   - MAPPs assay

3. CD4 T cell activation
   - DC internalization assay
   - DC activation assay

4. B cell activation & differentiation
   - B cell proliferation/differentiation assay
   - PBMC T cell proliferation assay
   - DC-T cell proliferation assay

Addition of peptides to HLA II antigens activates T cells and interacts with B cells to produce plasma cells and memory B cells.
An assay-suite can be applied to guide molecular design and lead selection

- Starting clones
- In silico prediction
  - De-select clones with high T cell epitope content and remove epitopes
- Synthetic 15-mer peptides
- PBMC:peptide T cell proliferation
  - Assess the T cell risk of parental and de-immunized variant sequences
- Full length proteins available
- MAPPs
  - Identify which peptides are presented by APCs
- Toxicity studies quality material available
- DC activation
  - De-select on DC activation risk
- Toxicity studies quality material available
- DC-T cell proliferation
- Final comparison of leads
  - Identification of lead with lowest immunogenicity risk
- Toxicity studies quality material available
- Synthetic 15-mer peptides
- Full length proteins available
- MAPPs
- DC activation
- Final comparison of leads
  - Identification of lead with lowest immunogenicity risk
DC activation assay principle & output: Pre-FFP validation

**PRINCIPLE**
Monocyte-derived DCs are grown from healthy donor cryopreserved PBMCs, incubated with test article and increase of activation markers is measured by flowcytometry

**READOUTS**

Stimulation Index (SI) = Test article treatment response / unstimulated control

\[
SI = \frac{TA(\%)}{BC(\%)}
\]

Positive response: \(SI \geq 1.4\)

Donor response frequency = Number of donors with positive response for a test article / Total number of donors x100

\[
RF(\%) = 100 \sum_{i=1}^{n} 1(SI_i > 1.4) / n
\]

**OUTPUT**
Risk ranking using donor response frequency; categories based on clinical relevance benchmarking

- Response frequency \(\leq 20\%\): Low risk
- Response frequency \(>20-50\%\): Medium risk
- Response frequency \(>50\%\): High risk

Flow cytometry profiles:
- Unstimulated control (No test articles added)
- KLR stimulated

Flow cytometry Readouts:
- Identify DC: CD11c
- 3 Flow cytometry readouts for DC activation:
  - % CD86^+ CD11c^+ DCs
  - % HLA-DR^+ CD11c^+ DCs
  - % CD40^+ CD11c^+ DCs

- 10 healthy donors
- Samples were tested in triplicates

PF-1 low clinical immunogenicity therapeutic
The main objective of a fit-for-purpose validation is to characterize key parameters and assess performance of an assay

- Quantify assay precision (intra-assay; inter-assay; inter-analyst)
- Understand and control the contributing factors to assay signal variability
- Establish a positive response threshold
- Determine the minimum required donor cohort size
- Determine in-study donor acceptance criteria
- Establish a robust data reporting approach
- Track assay performance and quality over time
Key parameters characterization and assay performance can be derived from a controlled precision assessment study

**Precision assessment**

<table>
<thead>
<tr>
<th>Intra-assay</th>
<th>Inter-assay</th>
<th>Inter-analyst</th>
<th>Inter-donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 experiment</td>
<td>3 independent experiments</td>
<td>1 independent experiment</td>
<td>1 experiment</td>
</tr>
<tr>
<td>1 analyst</td>
<td>experiments on different days</td>
<td>by second analyst</td>
<td>21 donors</td>
</tr>
<tr>
<td>6 replicates</td>
<td>Same analyst</td>
<td></td>
<td>1 analyst</td>
</tr>
</tbody>
</table>

4 test conditions covering a range of responses
- No stimulation: Background control
- KLH: High, System Control
- Bococizumab: Medium*, Therapeutic Control
- TAM-163: Low*, Therapeutic Control

*Clinical ADA incidence previously characterized
Using median value of sample triplicates, the positive response cut-off is set at $SI \geq 1.4$

Sample triplication provides balance between precision and throughput

Use of median obviates the need for pre-determined acceptance criteria for identification of outliers

With $SI \geq 1.4$ a test article gives a true response above background with 1% false positive rate
The overall SI precision meets the targeted %CV ≤ 30

DC Activation Assay SI Precision Data Distribution
Factors contributing to variability are suitable to enable test article differentiation using SI

Factors contributing to DC activation assay variability
Establishment of in-study donor acceptance criteria

• Data set: 21 donor panel from the validation + pre-validation experiments
• Performance factors considered:
  - Background DC activation
  - Response to System Control (KLH)

• Required for acceptance
  - Background activation ≤ 30%
  - System Control SI ≥ 1.4 for all markers

- Historical data demonstrate ~20% donors do not meet these acceptance criteria, hence each study is run with 15 donors to enable 10 reportable donors per study
Refined DC activation assay principle & output

**PRINCIPLE**
Monocyte-derived DCs are grown from healthy donor cryopreserved PBMCs, incubated with test article and increase of activation markers is measured by flowcytometry.

**READOUT**
- **Stimulation Index (SI)** = Test article treatment response/ unstimulated control
- **Positive response**: SI ≥ 1.4

**DONOR ACCEPTANCE CRITERIA**
- Donor response to KLH:
  - Background activation ≤ 30%
  - Positive response: SI ≥ 1.4 for all markers

- **Donor response frequency** = Number of donors with positive response for a test article/ Total number of donors x100

- Test 15 donors to report a minimum of 10 samples tested in triplicates
- Median value

**OUTPUT**
- Risk ranking using donor response frequency; categories based on historical data evaluation for clinical relevance
- Response frequency ≤20%: Low risk
- Response frequency >20-50%: Medium risk
- Response frequency >50%: High risk

- Median value PF-1 low clinical immunogenicity therapeutic
The sensitivity of the assay to TLR activation could allow its use for immunogenicity risk of generic peptides impurities

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity</th>
<th>Control peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC Activation</td>
<td>3pM</td>
<td>Pam3CSK4</td>
</tr>
</tbody>
</table>

Informed impurity test concentration

0.3 µM, and/or 3µM
Conclusion and outlook

The DC activation assay performance satisfies its intended use as an immunogenicity risk assessment screening tool for molecular design and lead selection of protein drugs

- Learnings from this FFP validation are being applied to other in vitro assays such as DC-T or PBMC:peptide T cell assays
- Further analysis of data continues to increase confidence in the tools
- The same FFP validation could be applied to the DC activation assay for intended use as an immunogenicity risk assessment tool for generic peptides
- A desirable next step is harmonization of methods across the field to allow comparability of results across laboratories. Working groups, such as the European Immunogenicity Platform’s Non-Clinical Immunogenicity Risk Assessment working group (NCIRA) are discussing strategies and recommendations for such harmonization
Acknowledgment

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Fit-for-Purpose Validation and Establishment of Assay Acceptance and Reporting Criteria of Dendritic Cell Activation Assay Contributing to the Assessment of Immunogenicity Risk.

Wickramarachchi D, Steeno G, You Z, Shaik S, Lepsy C, Xue L
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Thank you