Comparison of three cytokine release assays (CRA) for hazard identification of cytokine release syndrome potential of monoclonal antibody (mAb) therapeutics

Madeline Fort

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Cytokines are involved in many different immune responses and can be observed in patients given mAb therapeutics.

From Gribble et al., 2007. Expert Opin. Drug Metab. Toxicol. 3: 209-234
There are several known acute immune responses, but this discussion is focused on Cytokine Release Syndrome

- **Serum sickness**: delayed hypersensitivity reaction (~4-10 days) consisting of immune complex generation and vascular damage

- **Tumor lysis syndrome**: Rapid breakdown of lymphoma/leukemia cells results in biochemical abnormalities that can cause kidney damage and acute renal failure.

- **Allergic response (anaphylactic and anaphylactoid)**: hypersensitivity reaction consisting of IgE-dependent or independent mast cell degranulation.

- **Vascular Leak Syndrome**: increased vascular permeability resulting in decreases vascular resistance and interstitial edema in response to IL-2 treatment.

- **Cytokine Release Syndrome (CRS)**: Rapid, uncontrolled hypercytokinaemia that results in a range of clinical effects from pyrexia and fatigue to multiple organ failure
What makes CRS unique among acute infusion reactions?

- CRS can be associated with therapeutics whose mechanism of action involves targeted activation of immune cells
  - Can be associated with mAb which target receptors expressed on immune cells
  - Elevation of both pro-inflammatory (e.g. TNF-α) and anti-inflammatory (e.g. IL-10) cytokines.
  - Demonstrates dose dependency
  - Can be associated with tumor burden, target expression level or target cell abundance
  - Does not involve mast cell degranulation
Lessons learned from mAb therapeutics that cause CRS in the clinic: OKT3, alemtuzumab, and TGN1412

- Identification of potential CRS hazard is crucial prior to First-in-Human dosing
  - Onset of clinical symptoms: typically within 90 minutes of initiating treatment on first (and possibly subsequent) dose(s)
  - Prophylactic treatment with corticosteroids is more effective than treatment after onset of symptoms (at least for OKT3)

- Cytokine release due to exposure to a mAb involves a specific cell-cell interaction that should be amenable to in vitro detection prior to dosing patient

- Both Fab and Fc functions can be important for a mAb to induce CRS: screening assays need to probe both Fab and Fc functions
  - Data from anti-CD3 agonist mAb (OKT3 and visilizumab literature)

- Some cytokines of concern: TNF-α, IFN-γ, IL-6, IL-2
Streamlining CRS-prediction: Possible scenarios

**Unified Approach**
- All appropriate mAbs run through 1 or 2 assays
- Allows for consistency in assessments across programs
- Can build up experience with one or more assays and confirm/deny predictability
- Allows regulatory agencies to gain confidence in our approach
- Difficult to build one assay that can detect all mechanisms of CRS potential

**Specialized Approach**
- Create assays specific for the mechanism of a particular therapeutic mAb
- Allows for confidence that mAb mechanism of action is being directly assessed
- Requires extra time and resources to qualify new assays
- Will take longer to build up experience with each particular assay
- Must educate regulatory agencies on each assay
Expectations for a human cytokine release assay (CRA) for CRS prediction

- Must be relatively high throughput and detect cytokines previously observed with CRS-associated mAb therapeutics
  - TGN1412
  - OKT3
  - Alemtuzumab (Campath®-1H)

- Be able to detect cytokines in response mAb therapeutics w/ a weak or rare association with CRS in patients?
  - Trastuzumab (Herceptin®): known to cause mild-moderate first infusion reactions w/ pyrexia and fatigue. Not associated with clinically significant CRS.
  - Rituximab (Rituxan®): typically mild-moderate first infusion rxns; rare serious CRS have been documented; measurable TNF-α and IL-6 in serum of patients w/ high tumor burden.

- Must not give false positive results: mAb therapeutics not associated w/ CRS should be negative in the assay
  - Bevacizumab (Avastin®)
  - Infliximab (Remicade®)
  - Anti-streptavidin: in-house control human IgG2
### Human Cytokine Release Assays: comparison for detection of clinically significant CRS

<table>
<thead>
<tr>
<th>Assay</th>
<th>Positive mAb</th>
<th>Key Citations</th>
<th>Tested in-house</th>
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<tbody>
<tr>
<td><strong>Whole Blood Assays: soluble mAb/protein</strong></td>
<td>OKT3, alemtuzumab; CD28 superagonists;</td>
<td>Walker et al., 2011; Wolf et al., 2012.</td>
<td>No</td>
</tr>
<tr>
<td><strong>protein A beads</strong></td>
<td></td>
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<tr>
<td><strong>Solid Phase: dry coating of mAb to wells</strong></td>
<td>TGN1412, OKT3, alemtuzumab, trastuzumab, rituximab</td>
<td>Stebbings et al. 2007; Findlay et al. 2010.</td>
<td>YES</td>
</tr>
<tr>
<td><strong>Solution Phase: high density PBMC pre-culture</strong></td>
<td>TGN1412</td>
<td>Römer et al. 2011.</td>
<td>YES</td>
</tr>
<tr>
<td><strong>Co-culture system with HUVEC</strong></td>
<td>TGN1412, OKT3, alemtuzumab</td>
<td>Findlay et al. 2011; Dhir et al., 2012.</td>
<td>YES</td>
</tr>
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</table>
Solid Phase: dry coating of mAb to wells

- Antibody adherence to tissue culture plate:
  - Add small volume antibody solution to each well.
  - Plates are left overnight in a class II laminar flow cabinet with lids removed to allow the solution to slowly evaporate.
  - After overnight drying, wash all wells twice to remove salt crystals and any unbound antibody.
- Addition of human PBMC (125,000 cells/well): 16-24 hr incubation
- Remove cell supernatants to assay for cytokine production:
  - IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IL-12p70 using Luminex platform: Millipore Milliplex Human Cytokines (9 analyte panel)
Solid Phase CRA: cytokine production from mAb therapeutics with strong and weak CRS association

- Reproduced published results: alemtuzumab, trastuzumab, OKT3, rituximab

- **Concern for false positive responses:** infliximab and anti-streptavidin IgG2 induced pro-inflammatory cytokines in this assay, including TNFα, IL-6, IL-8, and IL-1β

Data representative of 3 separate experiments

*Anti-SA = anti-streptavidin human IgG2*
Solution Phase: high density PBMC pre-culture (Römer et al., 2012)

- Culture human PBMC at high density (10^7 cell/ml) for 48 hrs/37°C.
- After 48 hrs, wash and re-culture cells at 2 x10^5/well in a 96-well plate for 24 hrs.
- mAb tested:
  - TGN1412: 1µg/mL
  - alemtuzumab: 10µg/mL
  - rituximab: 10 & 100 µg/mL
  - trastuzumab: 10 & 100 µg/mL
  - OKT3: 1µg/mL
  - Anti-SA IgG2: 100 µg/ml
High density PBMC pre-culture CRA: cytokine release in response to soluble TGN1412 and OKT3

- Reproduced published results: soluble TGN1412 induced release of IFNγ, TNFα, IL-2, IL-4, IL-6, and IL-10

- No cytokine release with rituximab, trastuzumab, or anti-SA IgG2

Data from 5 human donors (denoted by color)

Anti-SA = anti-streptavidin human IgG2

Data representative of 4 separate experiments
Co-culture Platforms: human peripheral blood immune cells + endothelial cells

• Human umbilical cord vein endothelial cells (HUVEC) can provide necessary cross-linking for TGN1412 activation of human T cells
  • Original observation: Stebbings et al., 2007
  • Findlay et al 2011: HUVEC + human PBMC
  • Dhir et al. 2012: HUVEC + human peripheral blood leukocytes

• Human Immune Response Assay (HIRA):
  • In-house at Amgen
  • Culture of PBL (lymphocytes, monocytes, granulocytes) in 80% autologous platelet-poor plasma in the presence of HUVEC
  • 20-24 hr culture
  • TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10
HIRA: detection of pro-inflammatory cytokines in response to mAb therapeutics with strong CRS association in patients

- **OKT3 and CD28 agonist mAb:** induced T cell-associated cytokines including IL-2, IL-4, IL-10, as well as IFN-γ, TNF-α, IL-6, and IL-8
- **Alemtuzumab** induced cytokines associated with innate immune cells (NK cells, monocytes): IFN-γ, TNF-α, IL-6, and IL-8

Data from 6 human donors (denoted by color)
Anti-SA = anti-streptavidin human IgG2
HIRA: Cytokine release responses to mAb therapeutics are concentration-dependent

Data from 4-5 human donors (denoted by color)
Anti-SA = anti-streptavidin human IgG2
HIRA: no cytokine release in response to mAb therapeutics with weak or rare CRS association

No changes in IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, or TNF-α in response to infliximab, trastuzumab, or rituximab over a 100 fold concentration range.

**Anti-SA** = anti-streptavidin human IgG2

Data from 6 human donors (denoted by color)
PBL-HUVEC Co-culture is predictive for clinically significant CRS

<table>
<thead>
<tr>
<th>Platform</th>
<th>Pro-inflammatory Cytokines Detection</th>
<th>Comments</th>
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<tr>
<td></td>
<td>mAb with strong CRS association</td>
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<tr>
<td>Solid Phase</td>
<td>YES</td>
<td>YES</td>
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<td></td>
<td>YES</td>
<td>YES</td>
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<tr>
<td></td>
<td>YES</td>
<td>Concern for false positives</td>
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<tr>
<td>High Density PBMC Pre-culture</td>
<td>YES</td>
<td>NO</td>
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<tr>
<td></td>
<td>NO</td>
<td>NO</td>
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<tr>
<td></td>
<td></td>
<td>Specifically enhances detection of T cell activation; licensing fee</td>
</tr>
<tr>
<td>PBL-HUVEC Co-culture</td>
<td>YES</td>
<td>NO</td>
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<tr>
<td></td>
<td>NO</td>
<td>NO</td>
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<tr>
<td></td>
<td></td>
<td>Contains all circulating immune cells of interest</td>
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</table>
HIRA CRA is considered optimal for Amgen’s goal of identifying molecules with potential for clinically significant CRS risk

- Hazard identification approach for novel mAb and other Fc-bearing molecules
  - Final cytokine panel: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, TNF-α
  - ≥ 3X increase above background (PBL + HUVEC) levels of any cytokine is considered a “positive” for cytokine release
    - Cut-off based on data from negative controls (infliximab, anti-streptavidin, isotype controls)
  - OKT3 (positive control) used to confirm assay functioned as expected
  - No attempt to rank molecules based on cytokine levels: no comparison to positive controls used in the assay.
    - For proposed antagonist mAb candidates: any cytokine release above cut-off is considered a red flag
    - For proposed agonist mAb candidates: cytokine release is confirmation of mechanism of action and concentration-response curve may inform clinical dosing levels
HIRA at Amgen

- Protein therapeutics which target immune cell receptors and contain an Fc moiety are tested in HIRA
  - mAb for soluble targets or targets not expressed on immune cells are not routinely tested
  - Test a range of concentrations that are considered relevant to anticipated clinical exposures and/or to demonstrated on-target activity based on in vitro or preclinical in vivo studies.
  - Typically test 5-6 human donors simultaneously: logistically difficult to test more at this time
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