Adding Value to Cytokine Release Assays By Combining Analytical Techniques and Enhanced Data Analysis

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Introduction

Induction of cytokines, termed 'cytokine storm' is a common consequence of the administration of therapeutic antibodies. The worst occurrence being in 2006 when the administration of TGN1412 to subjects in a Phase I trial resulted in unprecedentedly high levels of cytokine release, leading to hospitalization of the subjects with systemic organ failure. This has led to a drive to produce ex vivo assays capable of indicating whether a novel therapeutic antibody would present a significant risk for cytokine release. Such assays have become widely used tools in early stage drug candidate screening, and are integral to the CTA process.

We present data from antibodies suitable to use as comparative controls for novel candidate drugs as they elicit a range of cytokine responses. We have combined measurement of the most commonly evaluated cytokines with ex vivo cellular analysis and demonstrate a novel data analysis approach for the often large and multi-parameter datasets generated.

Materials & Methods

Negative & Positive Control Antibodies

Palivizumab (Synagis), a humanized monoclonal antibody (IgG) against the F protein of respiratory syncytial virus (RSV) and not expected to bind to leukocytes.
Trastuzumab (Herceptin), a humanised antibody against HER2/neu, a member of the EGFR family and not expected to bind to leukocytes.
Alemtuzumab (Campath), a humanized monoclonal antibody (IgG) against CD52, a protein present on the surface of mature leukocytes.
YTH12.5, a rat IgG2b against CD3ε, expressed on T lymphocytes.
Muromonab (OKT3), a murine monoclonal IgG2a antibody against CD3ε, expressed on T lymphocytes (used in Method 2 only).

Method 1

Ex vivo Stimulation

Whole blood samples were collected into sodium heparin, to which control antibodies diluted in normal saline (0.9% NaCl) were added. Vehicle (negative) control wells were also set-up for each blood sample. Following incubation in a humidified 5% CO2:95% air atmosphere, at 37°C for 24 hours plasma fractions were collected and analysed for IFNγ, IL 1β, IL-2, IL-6, IL-10 and TNFα using a Luminex® analyser and MILLIPLEX MAP Human Cytokine/Chemokine Panel (Cat. No. HCYTOMAG-60K) in accordance with the kit manufacturer's instructions. The cell pellets were harvested and processed for flow cytometry analysis.

Flow Cytometry

Erythrocyte depleted whole blood samples were stained using LIVE/DEAD® Fixable Aqua stain (Invitrogen), followed by staining with lineage-specific, fluorochrome-conjugated antibodies (various vendors), as indicated Table 1. Samples were analysed using a 3-laser FACSCanto II™ flow cytometer (BD Biosciences).

Detector	Violet 450/50	Violet 510/10	Blue 530/30	Blue 585/42	Blue 670LP	Red 780/60	Red 680/20
Fluor	V450	BV510	FITC	PE	PECy5.5	PECy7	APC
Specificity	L/D Violet	CD15	CD14	CD25	CD4	CD8	CD69

Table 1. Panel descriptions
- Gating control panels for CD25 and CD69 are not shown

Method 2

Ex vivo Stimulation

Control antibodies, diluted in phosphate buffered saline were immobilised overnight onto either flat-bottomed polystyrene or round-bottomed polypropylene culture plates. Vehicle (negative) control wells were also set-up.

PBMCs were isolated from whole blood using sodium heparin Vacutainer® CPT™ cell preparation tubes (BD Biosciences). The cells were re-suspended in culture medium and incubated on the washed immobilised antibody surfaces for 24 hours, after which the supernatant fractions were harvested and analysed as indicated in Method 1.

Results

Method 1 Results

Ex vivo Stimulation

Whilst there was some inter-donor variation in responses, the general trends were clearly evident.

Cytokine Responses						
Stimulant (µg/mL)	IFNγ	IL-10	IL-1β	IL-2	IL-6	TNFα
VNC	8.3	5.1	<3.2	<3.2	7.1	9.3
StDev	9.6	2.8	0.5	1.8	4.2	4.7
Range	<3.2 - 33.1	<3.2 - 8.9	<3.2 - 3.6	<3.2 - 6.6	<3.2 - 14.4	<3.2 - 17.2
Synagis (1)	7.1	4.9	3.4	2.9	10.8	9.8
StDev	7.3	2.8	2.2	2.0	6.9	5.1
Range	<3.2 - 23.9	<3.2 - 9.6	<3.2 - 9.6	<3.2 - 7.5	<3.2 - 21.5	3.3 - 21.8
Herceptin (1)	3.9	<3.2	<3.2	4.6	3.5	7.1
StDev	2.5	1.4	2.4	5.6	1.5	2.3
Range	<3.2 - 10.7	<3.2 - 6.3	<3.2 - 10.4	<3.2 - 18.5	<3.2 - 6.3	4.1 - 15.6
YTH12.5 (10)	74.6	144.5	6.8	17.4	85.8	58.7
StDev	49.7	120.0	4.1	21.5	45.5	37.3
Range	11.4 - 165.8	7.3 - 326.0	<3.2 - 13.9	<3.2 - 67.7	28.8 - 176.3	9.6 - 101.5
YTH12.5 (1)	70.5	95.1	3.4	13.7	42.5	49.6
StDev	51.0	109.5	2.1	12.2	45.4	49.5
Range	11.4 - 170.1	4.7 - 251.8	<3.2 - 8.9	<3.2 - 40.4	9.4 - 157.4	5.9 - 174.9
YTH12.5 (0.1)	11.7	12.5	2.5	4.0	6.8	10.9
StDev	7.0	17.6	1.1	2.5	3.8	4.5
Range	<3.2 - 24.8	<3.2 - 67.6	<3.2 - 5.3	<3.2 - 9.9	<3.2 - 12.1	5.4 - 16.9
Campath (10)	277.7	9.4	21.0	3.2	546.1	133.5
StDev	160.1	3.1	13.8	2.5	286.8	111.8
Range	94.8 - 581.9	4.7 - 15.9	4.9 - 42.0	<3.2 - 9.2	149.8 - 1,158.0	53.1 - 363.2

Table 2. Method 1 Cytokine Response Data Summary

YTH12.5 (anti-CD3ε) induced a dose-related release of IFNγ, IL-10, IL-6 and TNFα and some low level IL-2. Campath induced strong release of IFNγ, IL-6 and TNFα (Table 2, Fig. 1). These data for Campath support the original findings that a clinical first-dose response was predominantly for these 3 cytokines (Wing et al , 1996).

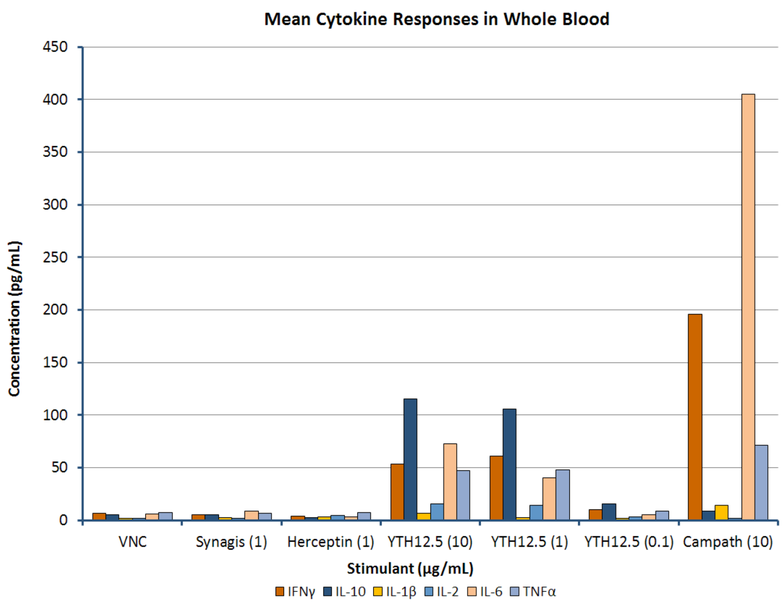

Figure 1. Mean Cytokine Response Trends

Flow Cytometry

Compared to the Synagis and the vehicle control, YTH12.5 induced increases in both CD25 and CD69 on all cell subsets, (Fig. 2a-d), the increases in CD69 being dose dependent. This was most marked on the CD4 and CD8 T cell subsets, in line with the target antigen of YTH12.5, but CD69 also increased on NK cells and monocytes, likely to be Fc-mediated.

Campath induced strong CD69 expression on NK cells, CD4 and to a lesser extent CD8 T cells and monocytes. Increased expression of CD25 was most notable on the NK cells and CD4 T cells (Fig. 2a-d). The observations for the NK cells Since Campath is reported to exert its main cytokine release in vivo from NK cells (Wing et al , 1996).

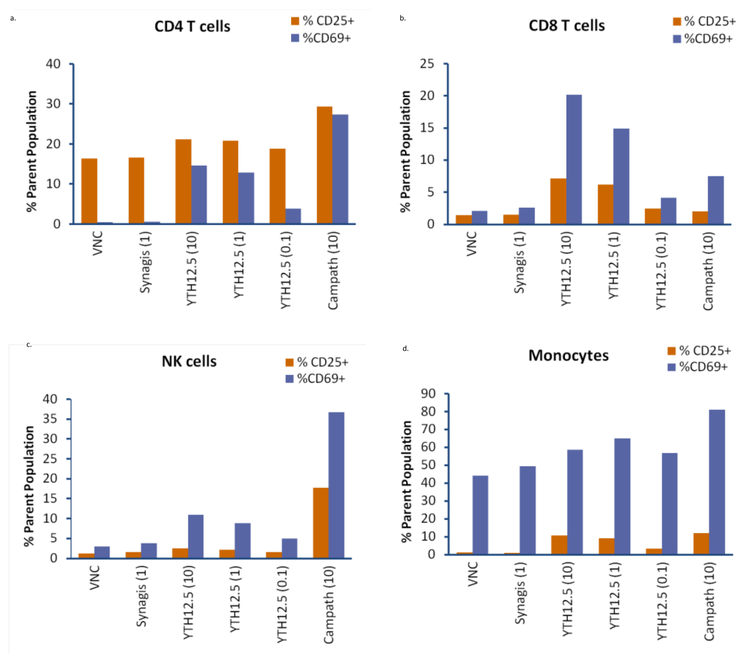

Figure 2. Cellular Activation Marker Data

These data show the utility of including an analysis of the cellular activation markers CD25 (IL-2R) and CD69 (AIM), differentiated into major cellular subsets, to provide additional information to a standard cytokine release assay and aid data interpretation.

Method 2 Results

When immobilised onto polystyrene plates the positive controls elicited a binary (on/off) cytokine response from the PBMCs; OKT3 consistently induced a strong response, whilst Campath showed stimulation of TNFα, but only at the highest concentration.

In contrast, when immobilised to polypropylene plates a more dose-related stimulation was observed. Again OKT3 elicited the strongest cytokine release for IFNγ, IL-6 and TNFα. This format appears to have a greater sensitivity as Campath induced higher TNFα and additionally also stimulated IL-6. In this format an additional antibody (Compound X) was shown to cause the release of all four cytokines, and the highest IL-2 levels (Fig. 3a-d).

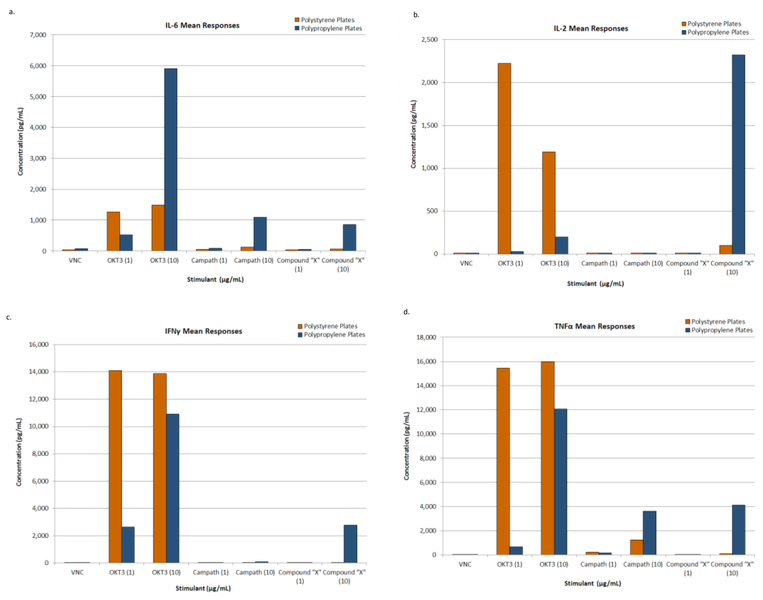

Figure 3. Substrate Impact on Cytokine Release Assays

Improved Data Analysis Approach

It is often difficult to discern the significance of cytokine stimulation data when presented as stimulation indices, or identify trends in the raw data from multiple donors. An approach that objectively evaluates the pattern of stimulation across all donors, taking analytical and biological variation into account is required. A cut-point approach, based on the distribution of the blanks could be taken, but necessitates the identification and exclusion of outliers, limiting the power of the assessment. An alternative approach, presented here is a simple non-parametric statistical evaluation. Stimulant pairs are compared using Wilcoxon Rank-Sum test, the null hypothesis being that there is no difference in cytokine production between the test item and the control. A 'box and whisker' plot summarising the distribution of plasma IFNγ responses for all donors and each stimulant at 10 µg/mL is shown here (Fig. 4).

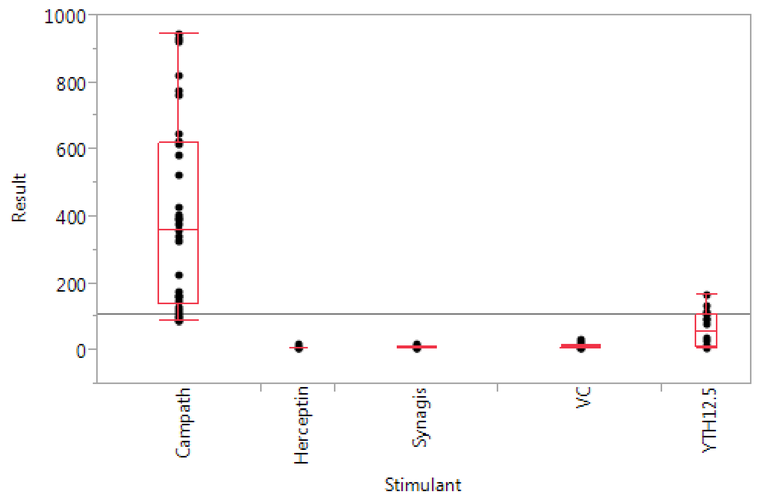

Figure 4. Box and whisker plot summary

The negative control Synagis (Fig. 5a) shows no significant IFN-γ production compared to the vehicle control (p=0.2372), and positive control Campath (Fig. 5b) is clearly significantly higher than Synagis (p<0.0001). Whilst there is an overlap between the α-CD3 YTH12.5 and Synagis (Fig. 5c), statistical evaluation confirms that the difference is also significant (p<0.0001).

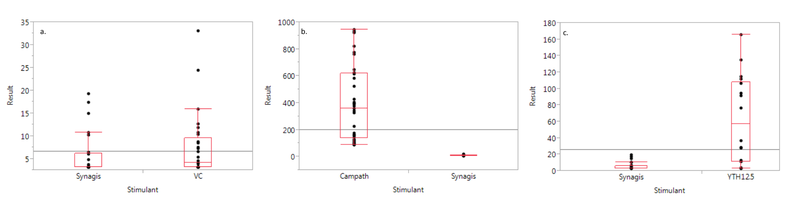

Figure 5. Wilcoxon pair-wise comparisons

Reference

M G Wing, T Moreau, J Greenwood, R M Smith, G Hale, J Isaacs, H Waldmann, P J Lachmann, and A Compston (1996) Mechanism of first-dose cytokine-release syndrome by CAMPATH 1-H: involvement of CD16 (FcgammaRIII) and CD11a/CD18 (LFA-1) on NK cells. J Clin Invest. 98(12): 2819–2826.

Summary

- The two positive control antibodies; YTH12.5 and Campath gave cytokine release data in line with their expected biological mechanisms of action. Whilst the vehicle control and two negative control antibodies; Synagis and Herceptin, showed no evidence of stimulating cytokine release.
- Data show the utility of including an analysis of the cellular activation markers CD25 (IL-2R) and CD69 (AIM), differentiated into major cellular subsets, to provide additional information to a standard cytokine release assay and aid data interpretation
- Ex vivo cytokine stimulation can give an indication of the potential for novel antibodies to induce CRS when administered.
- The understanding of the mechanism by which this occurs can be enhanced by evaluation of changes to the cell populations involved.
- Statistical evaluation of the results can offer an objective interpretation of the complex data sets generated