Validation of a serum-free approach to facilitate the development of high-throughput immunogenicity screening assays *in vitro.*

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Introduction

- The generation of anti-drug antibodies (ADA) towards a therapeutic agent can have severe implications for both drug safety and efficacy
- Classical *in vivo* models often fail to fully recapitulate the complexity of the human immune response and thus may not adequately predict the immunogenic potential of candidates entering the pipeline[1]
- As part of an early de-risking strategy, we present a high-throughput, *in vitro* approach to immunogenicity screening using two distinct models: i) peripheral blood mononuclear cells (PBMC) and ii) monocyte-derived dendritic cells (moDCs) isolated from the blood of healthy human donors
- In order to standardise our platform, we adopted a serum-free approach to cell culture which can be used to assess the immunogenicity of myriad modalities including biologics, oligonucleotides and small molecules

Method

- To investigate the impact of serum removal upon cellular viability, proliferation and cytokine release, PBMC and moDCs were cultured using a commercially available serum-free media (X-Vivo 15, Lonza) or RPMI 1640 supplemented with 10% human AB serum (serum-containing (SC) conditions)
- $CD14^+$ monocytes were seeded in 384-well plates and differentiated into moDCs using IL-4 & GM-CSF for 6 days prior to compound exposure. On day 6, moDCs were dosed with various concentrations of test articles in order to produce an 8-point dose response curve
- Following a 24hr incubation period, supernatants were collected via centrifugation and cells were assayed for viability (ATP content) using a CellTiter-Glo® luminescent assay (Promega). Cytokine analysis was performed upon collected supernatants using a multiplex cytokine bead array (BD Bioscience) according to the manufacturers instructions and data collected using an Intellicyt iQue 3 flow cytometer (Sartorius)
- For PBMC, cells were seeded in 384-well plates and exposed to compounds after a 24hr resting period. Lymphocytic proliferation and cell viability were measured in tandem after 72hrs using a resazurin based fluorescent assay (alamarBlue™, Thermo Fisher scientific) and CellTiter-Glo® kit. Supernatants were collected and analysed for cytokine release after 72hrs as previously described

Serum-free (SF) conditions improve the viability and morphology of moDCs in culture

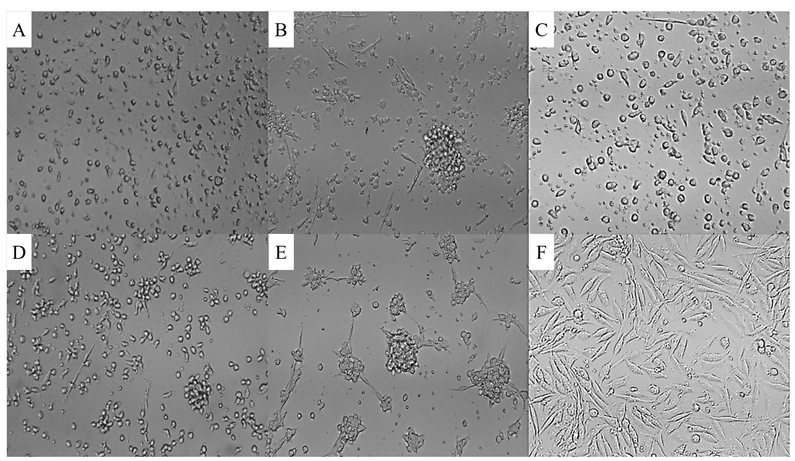

Figure 1: Brightfield images (40X mag.) illustrating the morphological differences between cells cultured in SC conditions (Figs. A-C) versus those cultured using SF conditions (Figs. D-F). Figs. A & D represent moDCs exposed to vehicle only (HBSS) while B & E and C & F highlight the typical phenotypic shift observed upon treatment with LPS (1µg/mL) and ODN 2395 (10µM) respectively for 24hrs. Evidence of cellular debris was reduced under SF conditions (Figs. A vs. D) and this was reflected by a moderate increase in ATP content (data not shown).

SF conditions enhance levels of cytokine release associated with PBMC proliferation while increasing the sensitivity of the plate-based lymphocytic proliferation assay

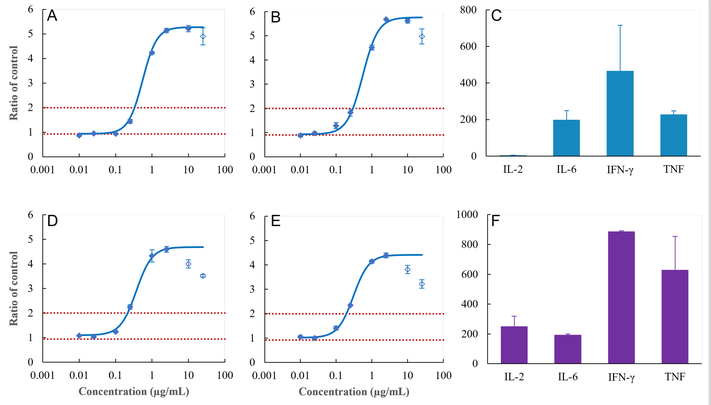

Figure 2: PBMC were exposed to PHA (0.01-25µg/mL) for 72hrs under SC (Figs. A-C) and SF conditions (Figs. D-F). Lymphocytic proliferation was assessed by measuring resazurin reduction (Figs B & E) and cellular ATP content (Figs A & D). Cytokine secretion was measured by flow cytometry using a multiplex CBA kit and the levels of IL-2, IFN-γ , IL-6 and TNF after exposure to 2.5 µg/mL PHA are shown (Figs. C & F). Red dotted lines represent both the lower solvent limit and an upper limit that conforms to the standard currently applied in assays used to evaluate compound immunogenicity (stimulation index (SI) – 2). Error bars show ±SD of triplicate intra assay replicates (n=1 donor). Unfilled circles indicate data points that were excluded from the final analysis.

- MEC values for lymphocytic proliferation generated under SF conditions were lower than those obtained from SC cultures (alamarBlue™ – 0.21 & 0.33 µg/mL respectively, ATP content – 0.19 & 0.29 µg/mL respectively)
- MEC values for IL-2 release were reduced in the absence of serum (0.25µg/mL vs 0.42 µg/mL) and levels were *ca* 60 fold greater under SF conditions

Measuring PBMC cytokine release and proliferation under SF conditions can be used to evaluate the immunogenic potential of various drug modalities *in vitro*

	Immunogenic *in vivo?*	IL-6	IL-2	IFN-γ	Proliferation
Biologics					
1. PHA	✓	↑ <0.004µg/mL	↑ <0.004µg/mL	↑ <0.004µg/mL	↑ <0.004µg/mL
2. Bevacizumab	ADA rate – 0%[3]	NR	NR	NR	NR
3. Adalimumab	ADA rate – 28%[3]	NR	NR	NR	NR
Oligonucleotides					
4. ODN2395 (Class C)	✓ - IL-6 secretion[1]	↑ 0.009µM	NR	NR	↑ 0.5µM
Small molecules					
5. CL075	✓	↑ 0.29µg/mL	↑ 0.62µg/mL	NR	↑ 3.1µg/mL

Table 1: MEC values derived from lymphocytic proliferation (alamarBlue™) and cytokine release data for various modalities including biologics (1-3), oligonucleotides (4) and small molecules (5). IL-2 and IFN-γ were selected as key markers of T cell activation while IL-6 was used as an indicator of a pro-inflammatory response[2]. PHA is a T cell mitogen and is known to stimulate the production of large quantities of IL-2 and IFN-γ. Bevacizumab and adalimumab are antibodies that target VEGF and TNF-α respectively and are generally considered to present a low –medium risk in terms of the generation of ADA in a clinical setting[3]. ODN2395 is an oligonucleotide that is known to stimulate significant IL-6 production via interaction with B cell and DC targets in vivo[4].CL075 is a TLR8 agonist that activates the NF-κB pathway culminating in the production of pro-inflammatory signals such as IL-6.[4] NR refers to a compound that is non-responsive in that feature of the assay and the arrow indicates the direction of the response.

SF moDCs can be used to evaluate both the toxicity and propensity of a compound to elicit a pro-inflammatory response at an early stage

	Immunogenic *in vivo?*	IL-6	TNF	IL-10	Cellular ATP
Biologics					
1. LPS EK	✓	↑ <0.0004µg/mL	↑ 0.001µg/mL	↑ 0.004µg/mL	NR
2. MDP	✓	↑ 0.01µg/mL	↑ 0.14µg/mL	NR	NR
Oligonucleotides					
3. ODN2395 (Class C)	✓	↑ 0.85µM	↑ 0.91µM	↑ 5.95µM	NR
4. ODN1668 (Class B)	✓	↑ 2.29µM	↑ 1.40µM	NR	NR
5. ssRNA40/LyoVec™	✓	↑ 0.13µg/mL	↑ 0.33µg/mL	NR	NR
Small molecules					
6. Resiquimod (R848)	✓	↑ 0.05µg/mL	↑ 0.29µg/mL	↑ 1.43µg/mL	NR
7. CL075	✓	↑ 0.06µg/mL	↑ 0.05µg/mL	↑ 0.32µg/mL	NR
8. Pindolol	×	NR	NR	NR	NR
9. Lapatinib	×	NR	NR	NR	↓ 4.65µM

Table 2: MEC values derived from cellular ATP and cytokine release data for various modalities including biologics (1-2), oligonucleotides (3-5) and small molecules (6-9). Compounds 1-7 are all known to be immunogenic whereas compound 8 has no known propensity to elicit an immune response *in vivo*. While lapatinib exhibits toxicity towards moDCs due to its chemotherapeutic nature, it has also been associated with delayed-onset hypersensitivity reactions; however, these are idiosyncratic and immune activation only occurs in susceptible individuals. NR refers to a compound that is non-responsive in that feature of the assay and the arrow indicates the direction of the response.

Conclusions

- Serum-free conditions provide a suitable environment in which to culture primary immune cells intended for downstream application in immunogenicity assays
- Eliminating serum enhances cytokine signal and assay performance without negatively impacting cell viability
- Measuring viability, lymphocytic proliferation and cytokine release in tandem provides the greatest mechanistic insight into the immunogenic affects elicited by candidate drugs
- Both moDCs and PBMCs can be used to accurately predict the likelihood of a pro-inflammatory response in a rapid, high-throughput format
- PBMC show greater sensitivity with regards to the prediction of a pro-inflammatory response where multiple cell types are involved in directing an immune response i.e. ODN2395

References

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