Tools for identifying and monitoring PD immune biomarkers from pre-clinical through to clinical stages of drug discovery.

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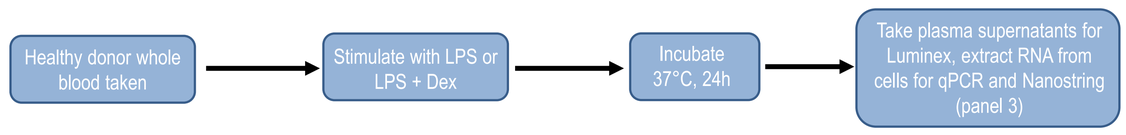
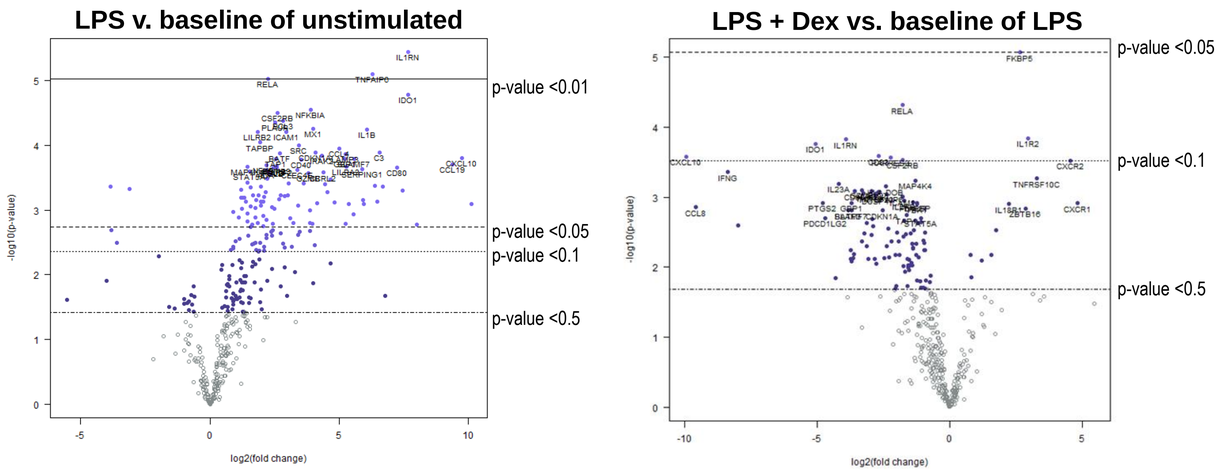
1 Introduction

Traditionally, primary and secondary endpoints in first-in-human clinical trials are concerned with safety. Expanding the remit of these studies to include exploratory biomarkers provides early information on efficacy and can confirm mode of action of the therapeutic in humans. Incorporating such endpoints from an early stage can also assist in go/no-go decisions and greatly increase the likelihood of success at progressing a therapeutic through the clinic. We have employed biomarkers to monitor immune responses and how these are modulated by novel therapeutics. To measure immune cell function ex vivo this often requires a stimulus, as early clinical trial are run in healthy individuals; here we show modulation of LPS-mediated stimulated immune responses as an example of translation of a biomarker assay throughout the stages of drug discovery. An *ex vivo* assay using LPS-stimulated human whole blood was used to screen a panel of ~800 genes using NanoString technology. A focused panel of informative biomarkers (IL-1β, IL-6, IFNγ and TNFα) was then taken forward to qPCR and multiplex Luminex assays, which confirmed the pattern of upregulation and reversal by dexamethasone. This was followed by an *in vivo* pharmacodynamic model of LPS-induced peritonitis using Luminex and flow cytometry readouts, which also showed good concordance. When a candidate compound progresses to first-in-human clinical trials, analysis of exploratory biomarkers is validated to Good Clinical Laboratory Practice (GCLP) standards to ensure reliable data. As such, the above Luminex analysis was validated to GCLP levels, where the performance of key assay parameters was determined. Thus, analysis of PD biomarkers in a translational manner provides early indications as to whether a therapeutic modulates its expected target when progressing into human trials.

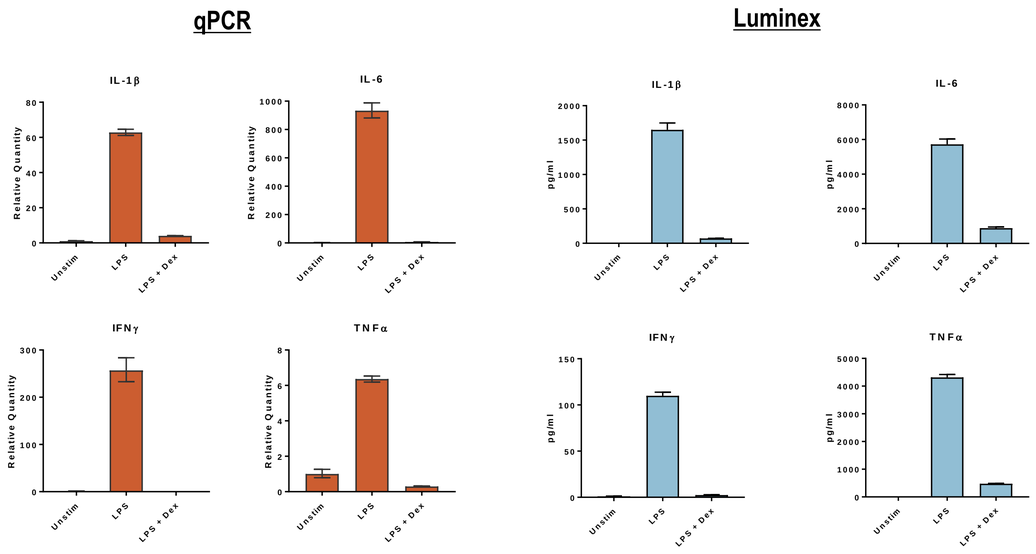
2 NanoString analysis of gene expression profile in stimulated whole blood identifies potential clinical biomarkers





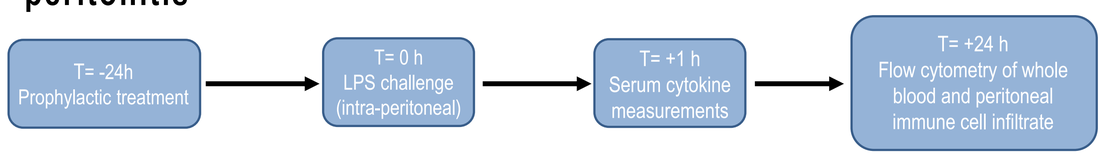
- Novel therapeutics can be screened in *ex vivo* whole blood assays using healthy donor blood stimulated through a relevant pathway to demonstrate target engagement and to identify potential new biomarkers.
- Gene expression profiling (up to 800 targets) performed using NanoString technology can be informative for biomarker selection. Horizontal lines indicate adjusted p value cut-offs. The further up the graph, the more statistically significant the change. Downregulated genes fall to the left, upregulated a genes are plotted further to the right.
- As expected, LPS induces upregulation of many genes (left), inhibition of LPS-driven inflammation with dexamethasone results in inhibition of gene upregulation or downregulation of many of these genes (right).

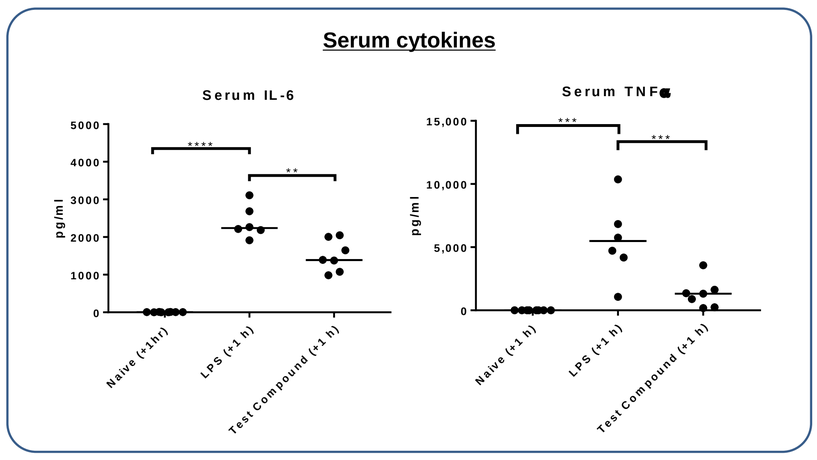
3 Confirmation and validation of potential inflammatory cytokine biomarkers by qPCR and Luminex

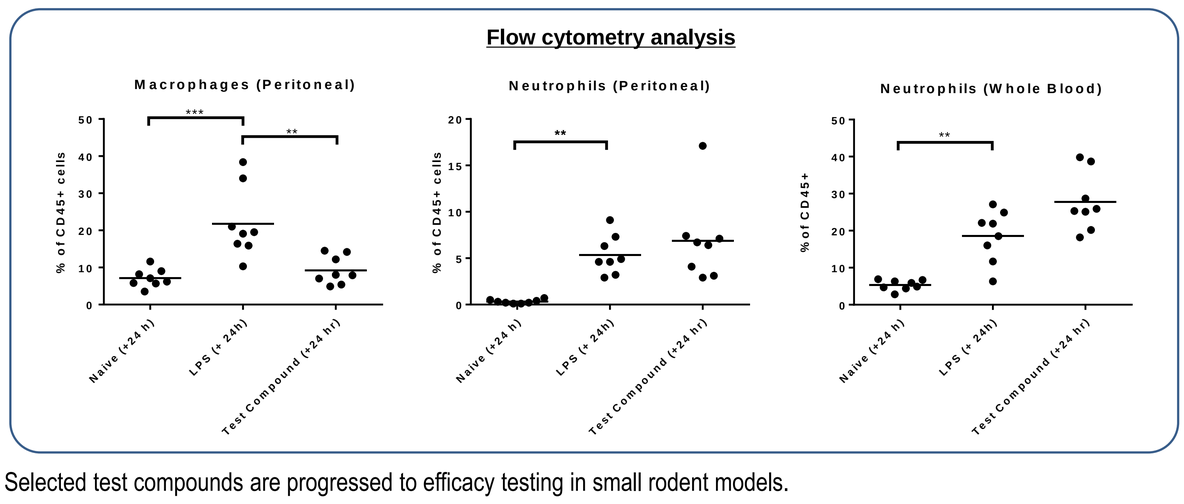


- Promising biomarkers, consisting of a more focused panel of analytes, are taken forward to confirm screening data.
- Inflammatory cytokine production can be screened at both the mRNA and protein levels by qPCR and Luminex, respectively.
- As illustrated here, the two techniques show good concordance for the cytokines tested.

4 *In vivo* pharmacology model to demonstrate efficacy – LPS-induced peritonitis







- Selected test compounds are progressed to efficacy testing in small rodent models.
- Analytical readouts include serum cytokine analysis and flow cytometry of peritoneal washes and whole blood.
- LPS challenge in mice triggers the release of pro-inflammatory cytokines IL-6 and TNFα, and an influx of macrophages and neutrophils into the peritoneal cavity. Levels of circulating neutrophils are also increased.
- The test compound demonstrates efficacy by reducing LPS-stimulated systemic cytokine levels, and by reducing the levels of peritoneal macrophages. Neutrophils are unaffected.

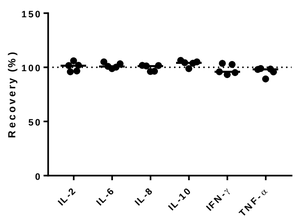
5 Exploratory end points supporting first-in-human clinical trials assist in critical decision making and provides information on candidate efficacy

Fit-for-purpose Validation
- Intra-assay precision
- Inter-assay precision
- Assay range (LLOQ, ULOQ)
- Recovery after spiking
- Recovery after long-term storage (mimic study logistics)
- Inter-operator variation

Inter-assay precision

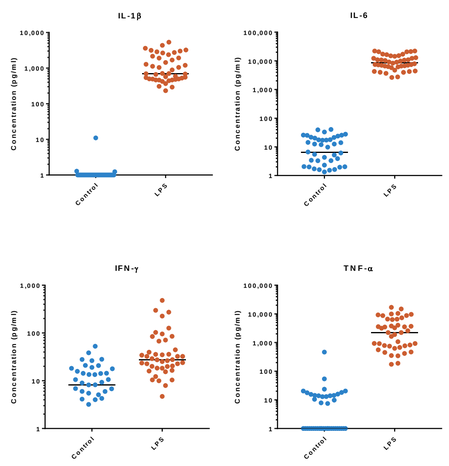
		Donor	TNF-α (pg/ml) Run 1	Run 2	Run 3	Average
24 h	Unstimulated	1	7.92	<LLOQ	<LLOQ	**7.92**
		2	<LLOQ	<LLOQ	<LLOQ	**N/A**
		3	9.37	8.24	7.91	**8.51**
	LPS, 10 ng/ml	1	5758.31	6115.79	5292.80	**5722.30**
		2	1174.94	973.56	870.47	**1006.32**
		3	1482.64	1454.15	1455.39	**1464.06**
	Activation QC (LPS)		3171.55	2833.74	2729.75	**2911.68**
	Plate QC (LPS)		3213.07	2776.71	2869.49	**2953.09**

Recovery from freeze-thaw (1 month)



Clinical Sample Analysis

Inter-donor variability



- Once a candidate therapeutic progresses to early phase clinical trials, measurement of exploratory biomarkers is validated before patient recruitment begins. Bespoke “fit-for-purpose” validation is performed for each trial, routinely to a higher level than is required for pre-clinical studies. During this validation phase, the trial logistics are mimicked to establish assay reproducibility.
- For example, depending on the trial logistics, samples may be collected in batches, stored and then analysed when a large enough pool of samples is available. Therefore, it is critical to know the biomarker recovery after storage. An understanding of inter-assay variability is also key.
- The graphs on the right show inter-donor variability in PBMC response to LPS stimulation in 45 healthy donors (activation on day of bleed, supernatants batched for measurement).

6 Summary

- Data shows that implementing efficacy biomarkers in early clinical trials reduces costs and increases success rates in the development of novel therapeutics.
- The Analytical Services group at Charles River Portishead specialises in developing biomarker assays that can be translated from pre-clinical through to early clinical studies.
- Inclusion of exploratory end points and biomarkers from the earliest possible stages of the drug development process can assist in go/no-go decisions, and provide crucial information on candidate efficacy.

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