Multiplex analysis of inflammatory cytokines from primary human macrophages using a FLUOstar® Omega

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- **Protein concentration of different inflammatory cytokines determined**
- **Downscaling from 96-well to 384-well without loss of sensitivity**
- **FLUOstar® Omega used to measure ELISA and Luciferase assays**

Introduction

Rheumatoid Arthritis (RA) is a chronic autoimmune disease characterised by chronic inflammation and the accumulation of immune competent cells in joints. The cells that accumulate in joints include macrophages and T cells and these cells produce inflammatory mediators, in particular Tumour Necrosis Factor (TNF), that drive the inflammatory process. These cells also show an increased yet insufficient production of anti-inflammatory cytokines including IL-10.

Recent work from this laboratory and others has shown in both human and animal models of RA that there is a potential role for the TLR (Toll like receptor) family in driving cytokine production in RA.

TLRs are receptors that recognise 'Pathogen Associated Molecular Patterns (PAMPs)' such as lipopolysaccharide (LPS) or dsRNA that are associated with bacterial, viral or fungal infections. The limitation for screening potential new targets has always been the availability of sufficient tissue/primary human cells. By reducing the number of cells required in an assay it would be possible to test more targets and multiple outputs on each donor. In this application note we show different assays to monitor cytokine activity on BMG LABTECH's FLUOstar Omega. By reducing the number of cells we downscaled from 96 well to a 384 well format.

The FLUOstar Omega is a versatile, automated micro-plate reader that offers a range of detection modes: UV/Vis absorbance spectra, fluorescence intensity, time-resolved fluorescence, time-resolved FRET, luminescence (flash and glow) and AlphaScreen®.

Materials & Methods

- Capture and detection antibodies for human TNF-α, and IL-6 were purchased from BD Pharmingen
- Macrophage-colony stimulating factor (M-CSF) was purchased from Peprotech
- LPS was purchased from Alexa
- 96-well clear tissue culture plates
- 384-well clear tissue culture plates, Appleton Woods
- 96-well and 384-well Luciferase assay plates with solid strips
- 96-well and 384-well ELISA plates, Appleton Woods
- FLUOstar Omega, BMG LABTECH

Cells and Cell Culture

PBMCs (Peripheral Blood Mononuclear Cells) were prepared from buffy coat fractions of a unit of blood from a single donor using Ficoll-Hypaque (Nycomed). Monocytes were isolated by centrifugal elutriation (>85% purity), routinely collected and cultured in RPMI medium containing 10% heat inactivated foetal calf serum (FCS), as previously described. After that monocytes were differentiated in the presence of 100 ng/mL M-CSF (Peprotech) for 3 days. Non-adherent cells were washed off and the remaining adherent cells were removed using cell dissociation media (Sigma), counted and re-plated prior to assay. 96-well plates have 5x the growth area of 384-well plates so cell number and volume of media has been reduced by same ratio. Cells are stimulated by the addition of 10 ng/mL *E.coli* derived LPS (Alexa). All cell culture incubations were performed at 37°C in a humidified atmosphere containing 5% CO_2.

Generation of Adenoviral Vectors and Cell Infection

Recombinant, replication-deficient adenoviral constructs were prepared using the AdEasy system as previously described. MCSF derived macrophages were plated in a 96-well plate at $1x10^5$ cells/well, $2x10^4$ cells/well in 384-well plate and allowed to express adenoviral transgenes for at least 24 hours. Adenovirus used is the NFκB luciferase adenovirus (AdNFκB-luc) and contains four tandem copies of the κ enhancer element located upstream of the firefly luciferase gene. This adenovirus was provided by P.B. McCray Jr. (University of Iowa, Iowa City, IA) and is a modification of the pNFκB reporter vector (BD Clontech).

ELISA

Macrophages were plated at $1x10^5$ cells/well in a 96-well plate and at $2x10^4$ cells/well in a 384-well plate, incubated for 24 h at 37°C, and stimulated with 10 ng/mL LPS. Supernatants were harvested after 18 h and examined for concentrations of TNF-α and IL-6 following the manufacturers' instructions. Absorbance was read at 450 nm on a FLUOstar OMEGA and analysed using the MARS data analysis software. Results are presented as the mean concentration of triplicate cultures ± SD.

Luciferase Reporter Gene Assay

Cells were infected with a range of Moi (multiplicity of infection i.e. number of virus particles/cell) to generate a standard curve for GFP expression and luciferase units. After LPS stimulation, cells were washed once in PBS and lysed with 100 µL (96-well) or 50 µL (384-well) CAT lysis buffer. Cell lysates (50 µL 96-well or 25 µL 384-well) were transferred into a luminometer cuvette strip and (120 µL 96-well or 60 µL) luciferase assay buffer and 30 µL luciferin added as described previously. Luciferase activity was measured by detecting luminescence in relative luminescence units (RLU) using the FLUOstar Omega reader and MARS data analysis software.

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Results & Discussion

After macrophage stimulation with LPS the concentration of TNF-α obtained in 96-well and 384-well format is comparable, as long as the ratio of cells and culture volume is kept constant to the growth area of the well (Fig 1).

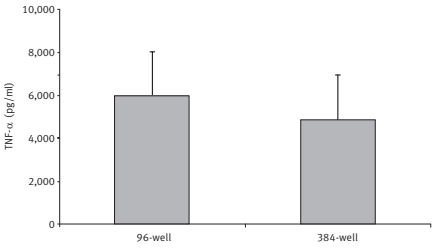

Fig. 1: Data from 3 donors comparing TNF-α concentration in supernatants from cells cultured in 96-well and 384-well plates.

IL-6 concentrations are also comparable between 96-well and 384-well plates (Fig 2).

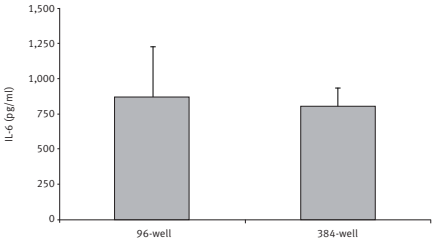

Fig. 2: Data from 3 donors comparing IL-6 concentration in supernatants from cells cultured in 96-well and 384-well plates.

ELISA measurements for quantifying further cytokines like IL-10 and IP-10 were also performed successfully in 384-well plates. The results were in accordance to the values obtained in the 96-well format (data not shown). Active cytokine NFκB was detected with the help of the luciferase assay (Figure 3).

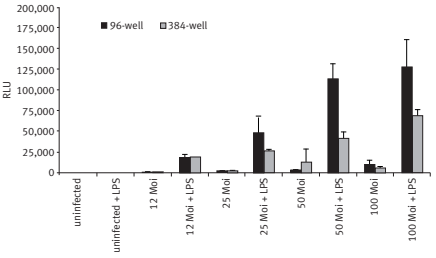

Fig. 3: Luciferase assay of human primary macrophages infected with NFκB Adenovirus in 96-well and 384-well format.

When using less than 25 Moi of virus sensitivity makes the assay unreliable.

The 96-well and 384-well results shown in Figure 4 are not comparable in their signal height as the 384 well plates were lysed in 2.5 fold more lysis buffer hence the relative luciferase units are approximately 2.5 fold lower than those obtained using 96 well plates. More lysis buffer was added to 384 well plates to ensure there was enough lysate to perform luciferase assay.

Conclusion

The FLUOstar Omega plate reader gives the potential to analyse multiple cytokines on the same assay plate when using the grouping feature. This pilot study shows that the human primary macrophages can be cultured in 384-well plates without any changes in morphology or cytokine expression.

Reducing the number of cells needed for each assay without altering the sensitivity will now open possibilities to do screening assays on primary cells to look for new targets in autoimmune diseases. Previously this has only been possible in cell lines that do not respond to TLR ligands in the same way as primary cells.

Successful Adenovirus infection of primary human macrophages in 384-well plates also allows reporter assays to be performed.