High Throughput Quantitation of Cytokine Biomarkers using LANCE® *Ultra* TR-FRET Assays

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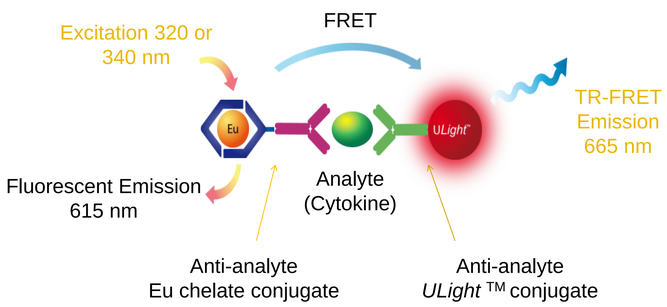
1 Introduction

Injury to tissue can lead to inflammation which is characterized by an accumulation of cytokines. The excessive production of pro-inflammatory cytokines can result in further tissue damage and has been implicated in many diseases such as atherosclerosis and cancer. Anti-inflammatory drug development is therefore an important area of focus in the pharmaceutical industry. Discovery of these drugs through high throughput screening campaigns requires the ability to both robustly and reliably detect cytokine production in a variety of complex sample matrices. Here, we demonstrate the ease and versatility of using LANCE *Ultra* TR-FRET assays to quantitate low levels of the pro-inflammatory cytokines TNFα, IL6, and IL1β, from differentiated U937 cell supernatants and detect measurable inhibition of cytokine secretion using the small molecule dexamethasone.

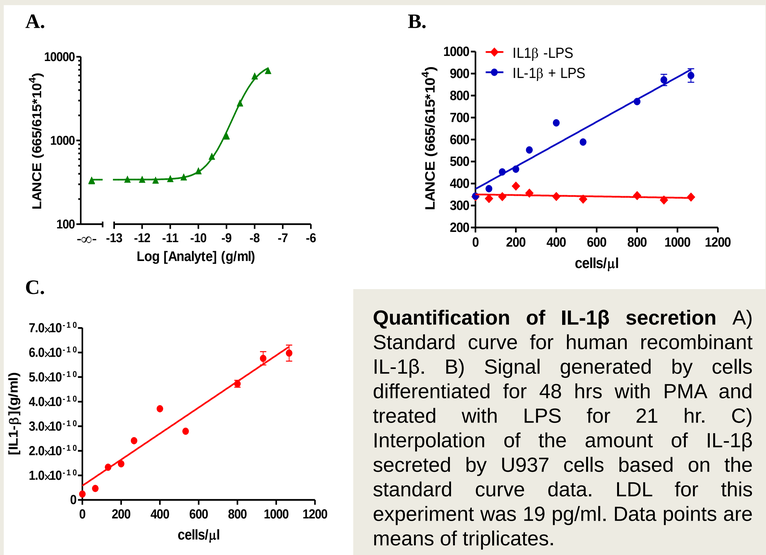
2 Assay and Methods



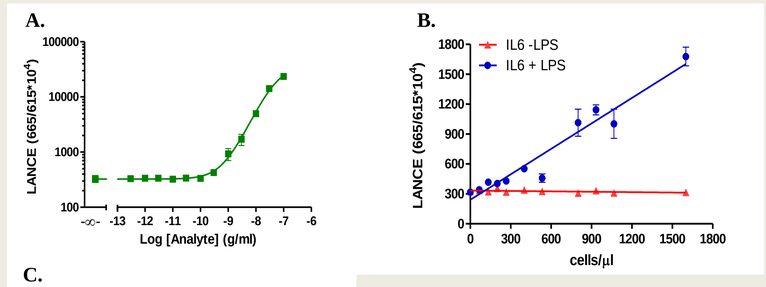
LANCE *Ultra* TR-FRET Assay: The cytokine in the cell supernatant is captured by a Europium-labeled antibody and a U*Light*™ labeled antibody to form a complex. Excitation of the Eu chelate in the complex generates signal at 615nm and after the energy transfer, signal is generated at 665 nm.

- U937 cells were differentiated for 48 hours with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL).
- In 2% FBS, challenged for 21 hours with lipopolysaccharides (LPS; 10 µg/mL) in 96-well CulturPlates.
- 15 µl of supernatants were transferred to a 384-well OptiPlate plates with 5 µl of a mixture of Eu-antibody and U*Light*™ antibody and incubated for 1 hour at room temperature.
- Signal was measured on an PerkinElmer EnVision Multimode Plate Reader Model 2104 (dual detectors and a laser)
- LANCE *Ultra* kits used are IL-1β (TRF1220C), TNFα (TRF1208C) and IL-6 (TRF1223C).
- For inhibition studies, U937 cells were differentiated with PMA for 48 hour and then with both LPS (10 µg/mL) and increasing concentrations of dexamethasone for 21 hours.
- For miniaturization studies, 7.5 µl (384-well ProxiPlate) or 3.75 µl (1536-well OptiPlate) of each concentration of cells were used in the assay. For 10 µl reaction in 384-well ProxiPlate, 2.5 µl of of Eu-antibody and U*Light*™ antibody were used. For 5 µl reaction in a 1536-well OptiPlate, 1.25 µl of Eu-antibody and U*Light*™ antibody were used.
- Data was plotted as the ratio of the emission of the acceptor (665 nm) and the emission of the donor (615 nm) against increasing analyte concentration.
- The lower detection limit (LDL) was calculated using 3 standard deviations from the mean of the background.

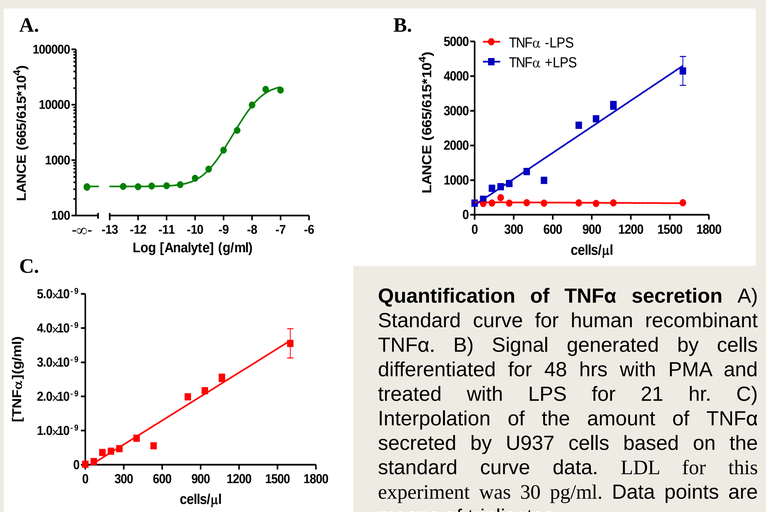
3 Cytokine Detection in Supernatants of Differentiated U937 Cells



Quantification of IL-1β secretion A) Standard curve for human recombinant IL-1β. B) Signal generated by cells differentiated for 48 hrs with PMA and treated with LPS for 21 hr. C) Interpolation of the amount of IL-1β secreted by U937 cells based on the standard curve data. LDL for this experiment was 19 pg/ml. Data points are means of triplicates.



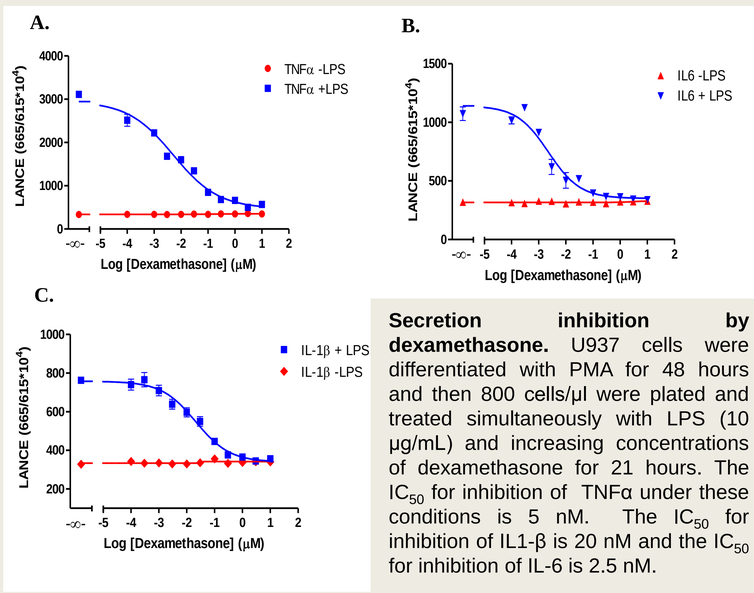
Quantification of IL-6 secretion A) Standard curve for human recombinant IL-6. B) Signal generated by cells differentiated for 48 hrs with PMA and treated with LPS for 21 hr. C) Interpolation of the amount of IL-6 secreted by U937 cells based on the standard curve data. LDL for this experiment was 93 pg/ml. Data points are means of triplicates.



Quantification of TNFα secretion A) Standard curve for human recombinant TNFα. B) Signal generated by cells differentiated for 48 hrs with PMA and treated with LPS for 21 hr. C) Interpolation of the amount of TNFα secreted by U937 cells based on the standard curve data. LDL for this experiment was 30 pg/ml. Data points are means of triplicates.

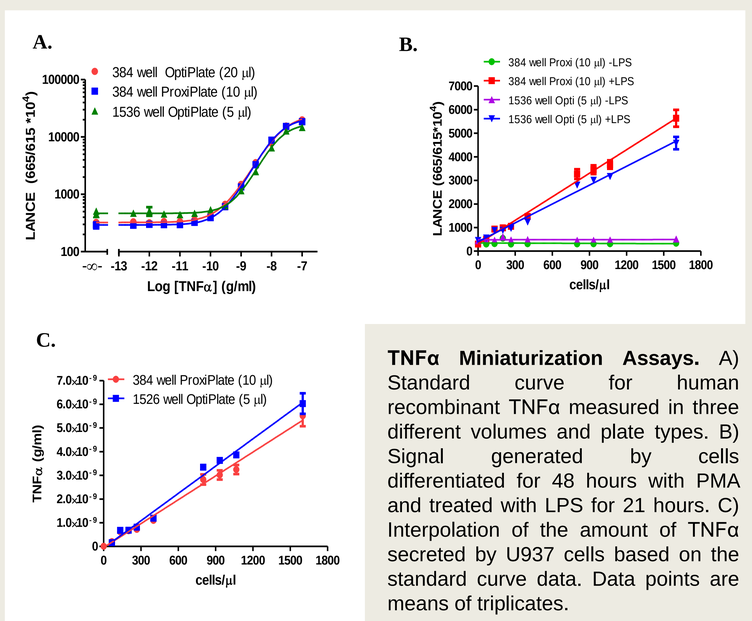
4 Cytokine Secretion Inhibition by Dexamethasone

The effect of the glucocorticoid dexamethasone on the secretion of IL-1β, TNFα, and IL-6 was determined using LANCE *Ultra* kits.



Secretion inhibition by dexamethasone. U937 cells were differentiated with PMA for 48 hours and then 800 cells/µl were plated and treated simultaneously with LPS (10 µg/mL) and increasing concentrations of dexamethasone for 21 hours. The IC_{50} for inhibition of TNFα under these conditions is 5 nM. The IC_{50} for inhibition of IL1-β is 20 nM and the IC_{50} for inhibition of IL-6 is 2.5 nM.

5 Assay Miniaturization



TNFα Miniaturization Assays. A) Standard curve for human recombinant TNFα measured in three different volumes and plate types. B) Signal generated by cells differentiated for 48 hours with PMA and treated with LPS for 21 hours. C) Interpolation of the amount of TNFα secreted by U937 cells based on the standard curve data. Data points are means of triplicates.

6 Summary & Conclusion

- LANCE *Ultra* TR-FRET kits for the detection of cytokine biomarkers successfully detect cytokines secreted by stimulated U937 cells in RPMI media
- Inhibition of IL-1β, TNFα, and IL-6 secretion by a known inhibitor of inflammation (dexamethasone) was demonstrated.
- Assay can easily be miniaturized to 10 µl volume in a 384-well Proxiplate or 5 µl in a 1536-well OptiPlate.