A Leukocyte Adhesion Assay Performed on BMG LABTECH's FLUOstar OPTIMA

BMG LABTECH

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- Leukocyte adhesion assay utilized to screen for potential inflammatory markers
- FLUOstar OPTIMA used for cell based bottom optic measurements and well scanning
- Results show good reproducibility and can be adapted to e.g. primary neutrophils

Introduction

A key component of the inflammatory response is the recruitment and extravasation of leukocytes into perivascular tissue. This process requires leukocyte interaction with vascular endothelium and consists of a multistep process including the capture of circulating leukocytes, subsequent leukocyte rolling, arrest, firm adhesion and transmigration[1] (Figure 1). This complex process initially involves the recognition and interaction of endothelial cell adhesion molecules with their specific ligands on leukocytes. The capture and the removal of the leukocytes from the flowing blood, as well as the subsequent rolling of the leukocyte along the vessel wall, are due to the reversible binding of selectins which are found on both the leukocytes and the endothelial cell surface. Arrest and firm adhesion of leukocytes to endothelium is dependent on the activation of β2 integrins including Mac-1 or LFA-1 on the leukocyte cell surface, followed by interaction with endothelial cell proteins belonging to the immunoglobulin superfamily such as ICAM-1[2].

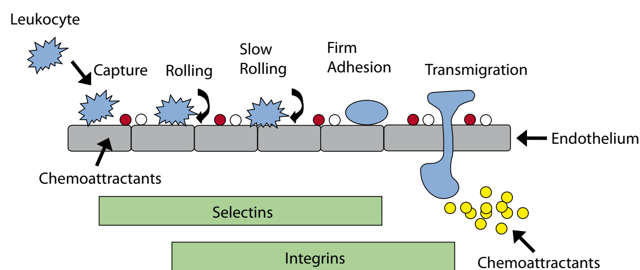

Fig. 1: Processes involved in leukocyte capture, adhesion and transmigration across the endothelium[1]

We have used gene regulatory networks to identify novel regulatory hubs genes involved in the inflammatory response in human umbilical endothelial cells (HUVECs). To assess the potential role of these genes we have used a leukocyte adhesion assay to model this complex process and looked at the response of the endothelium following RNAi knockdown of the selected genes. We have used primary peripheral blood mononuclear cells (PBMCs) and a number of leukocyte cell lines (HL60, JY and U937) which differ in their adhesion ligands.

Materials and Methods

The materials were purchased from the following manufacturers:

- FLUOstar OPTIMA, BMG LABTECH, Aylesbury, UK
- siRNA on-target reagents from Dharmacon, Perbio, Northumberland, UK
- Cell tracker Green dye from Invitrogen, Paisley, Scotland, UK
- Microplates, black 96-well from Greiner, Gloucestershire, UK
- Tissue culture media from Lonza, Wokingham, Berkshire, UK
- HL60.Ast and JY cell lines from ECACC, Porton Down, UK
- TNFα recombinant protein from R&D systems, Oxford, UK

Fig. 2: BMG LABTECH's FLUOstar OPTIMA microplate reader

HUVECs were grown in EGM2 culture media and were combined into pools of cells from 10 donors for each experiment. Cells were grown for 24hrs following recovery from frozen storage and plated into T25 tissue culture flasks. Transfections were carried out using 100 nM of siRNA on-target plus pools, 6 hrs after transfection the cells were re-seeded into 96-well plates.

To allow knockdown of the target protein the cells were left for 48 hrs post transfection in normal growth conditions. The plates were then gently washed using a multichannel pipette. EGM2 media +/- TNFα was added to selected wells. The plates were incubated for 4 hrs to allow upregulation of cell adhesion molecules, and then washed gently once with PBS.

Whilst incubating the HUVECs with TNFα, the HL60 cells (or other leukocyte cells) were labelled with cell tracker green (2.5 µM in PBS) for 25 min at 37°C following the manufactures instructions. After labelling and washing the HL60 were resuspended in EGM2 and then added to all wells ($1x10^5$/well) except the negative control wells to which unlabelled cells were added.

The plates were incubated for 1 hr to allow leukocyte adhesion and then washed gently x3 with PBS at RT to remove any unbound cells. Fluorescence in each well was then quantified using the FLUOstar OPTIMA (Excitation 485 nm and emission 520 nm) with replicates of 5 per sample. Knockdown of the TNFRSF1A[3] was used as a positive control.

Results and Discussion

Initial results demonstrated that there was a linear relationship between cell number and fluorescence (Figure 3).

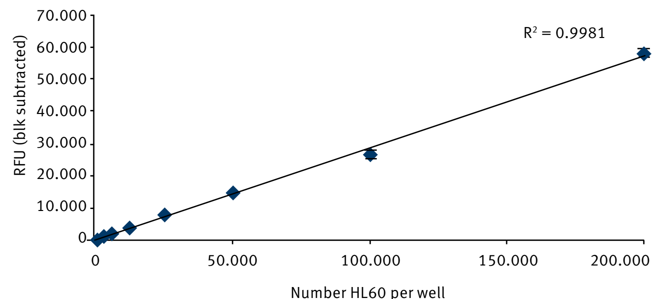

Fig. 3: Cell tracker green labelling of HL60 cell line showing linear relationship to cell number in 96 well plates

Preliminary adhesion assays were then set up using HUVECs that were transfected with siRNA for luciferase (siControl) and TNFRSF1A (positive control). 48 hrs after transfection the cells were treated with TNFα (0-10 ng/mL) for 4 hrs prior to the addition of cell tracker green labelled HL60 cells. Following 1 hr incubation and gently washing to remove unattached HL60, the fluorescence was quantified using BMG LABTECH's FLUOstar OPTIMA in fluorescence mode with bottom optic measurement. Results shown in Figure 4 demonstrate that increasing the concentration of TNFα causes increased adhesion of HL60 to the HUVEC monolayer in both untreated and siControl KD conditions. However following siRNA knockdown of the TNFRSF1A there is no significant effect of TNFα on HL60 adhesion. This would be predicted as this receptor is required for TNFα signalling.

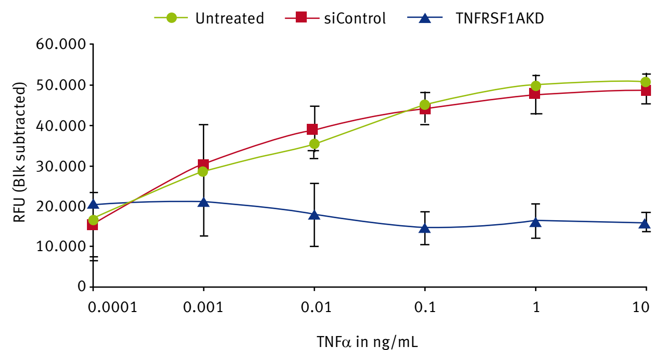

Fig. 4: Adhesion of Cell tracker green labelled HL60 cell line to TNFα treated HUVEC following siRNA gene knockdown (TNFRSF1AKD).

QPCR studies confirm that the knockdown efficiency was >90%. This is a very valuable positive control for this assay and was used for all subsequent studies. The well scanning mode of the FLUOstar OPTIMA is helpful when scaling this assay up to use in 48 well plates as it ensures accurate quantification due to the possible uneven distribution of the HL60 cells. This function also provides a good visual output for the assay in a 96-well plate as shown in Figure 5 below.

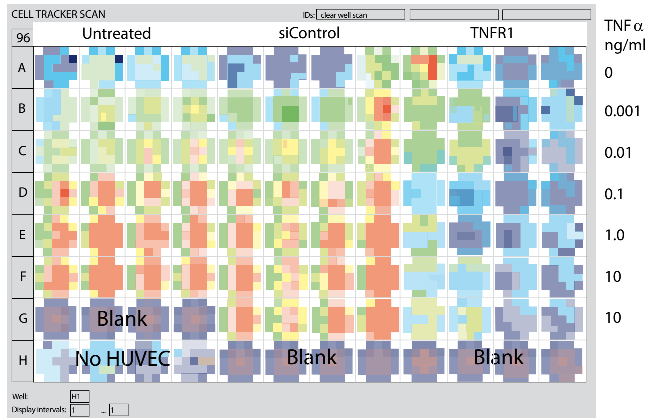

Fig. 5: Well scanning view of cell tracker green labelled HL60 adhesion to TNFα stimulated HUVEC. (Blank wells contain unlabelled HUVECs in media, the "no HUVEC" wells contain labelled HL60 to check adhesion to well surface)

The results were compared with fluorescent microscopy analysis of the wells (data not shown). This showed a very similar pattern but use of the microplate reader enables rapid analysis of multiple plates rather than the time consuming image capture and quantification by microscopy.

Conclusion

The results show good reproducibility for a complex biological assay and provide a method for rapid screening of this aspect of the inflammatory process. We have utilized this method to screen a number of potential inflammatory markers and it provides a very easy to use technique which can also be adapted for primary neutrophils or PBMC if a more "in vivo-like" assay is required.

References

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