

# Characterisation of THP-1 macrophages as an *in vitro* model for CD44-targeted therapies

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## ABSTRACT

CD44 is the major cell surface receptor of hyaluronic acid (HA) and mediates the response of cells to their microenvironment. Whereas the standard isoform (CD44s) is ubiquitously expressed throughout the body, variant isoforms (CD44v) are aberrantly overexpressed in certain tumours, thus CD44 has become a potential target for cancer chemotherapy. However, CD44 is also one of the most abundantly expressed macrophage receptors, rendering this cellular type a potential competitor for the uptake of targeted nanocarriers *in vivo*.

An *in vitro* human macrophage model is described herein. This model would allow to evaluate CD44 off-targeting. In particular, we have developed a procedure for the differentiation and activation of THP-1 cells. The CD44 expression of THP-1 macrophages was compared to that of the tumoural cell line HT-29 in terms of amount and isoform composition. The internalisation kinetics of HA nanoparticles was also evaluated.

**Keywords:** CD44s, CD44v, hyaluronic acid, CD44-targeted therapies, THP-1 macrophages

## 1 INTRODUCTION

CD44 is the major cell surface receptor of HA [1] and is increasingly recognised as one of the ‘hot’ receptors in targeted tumour therapy. The success of targeting therapies is hindered mainly by the complexity of the receptor. CD44 is exquisitely regulated at the post-transcriptional level resulting in a combination of standard (CD44s) and variant (CD44v) isoforms. Second, the different isoforms are susceptible to numerous post-translational modifications. Indeed it is well known that CD44s/v-HA interactions have a key role in tumorigenesis, metastasis, and putatively in the identity itself of cancer stem cells [1, 2]. However, CD44 is also widely expressed on other cells, such as macrophages resident in the organs of the reticuloendothelial system. Importantly, it has been reported that monocyte-derived macrophages express an

active (HA-binding) state of the receptor whereas freshly isolated peripheral blood monocytes express an inactive form [3]. Therefore, in view of the development of CD44-targeted therapies and foreseeing the intravenous injection of appropriate carriers, it is chief to assess possible off-targeting effects.

The main constraint when considering experimental work with human macrophages is their difficult isolation, the invasiveness of the isolation procedures, and their inability to proliferate in culture. For these reasons a number of studies focused on CD44-targeting make use of a well established murine cell model, RAW 264.7 macrophages [4] e.g. as a positive control for uptake experiments [5, 6]. Yet, the use of a murine cell line presents obvious limitations.

The human monocytic leukaemia THP-1 cell line shows commitment towards macrophage differentiation [7-9]. THP-1 macrophages resemble native monocyte-derived macrophages in terms of morphology, expression of membrane antigens and receptors, transient induction of several proto-oncogenes, and secretory products [7]. To the best of our knowledge, it is currently not known whether macrophages exhibit a specific CD44s/CD44v expression pattern, whether this depends on the degree and mode of activation, and if this might influence the affinity towards HA. The aim of the present study was to develop an *in vitro* human macrophage model to study the role of these sentinel cells in the uptake of CD44-targeting carriers.

## 2 MATERIALS AND METHODS

### 2.1 General Cell Culture

The human monocyte cell line THP-1 (TIB-202™) and the colorectal carcinoma cell line HT-29 (HTB-28™) were purchased from American Type Cell Collection (ATCC) (Manassas, VA, USA). Both cell types were maintained at 37°C in a humidified 5% (v/v) CO<sub>2</sub> air atmosphere. The medium was changed every 2-3 days as recommended by the supplier.

THP-1 premonocytes were cultured in RPMI 1640 medium (Gibco®/Invitrogen, UK) supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-Glutamine, 1%

(v/v) antibiotic-antimycotic solution and 50  $\mu\text{M}$  2-mercaptoethanol (Sigma-Aldrich, UK). Differentiation into THP-1 macrophages was triggered by incubation with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, UK) for 24 hours. Classical and alternative activation were achieved by treatment with LPS/IFN- $\gamma$  or IL-4/IL-13, respectively.

The human colorectal adenocarcinoma HT-29 cell line was cultured in Modified McCoy's 5A medium supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution (Sigma-Aldrich, UK).

Phase contrast images of live THP-1 macrophages were recorded with a Leica DM6000 B inverted microscope (Leica Microsystems, UK) coupled with a 5.5 Neo sCMOS camera (Andor, UK) and the  $\mu\text{Manager}$  software (v.1.46).

## 2.2 CD44 Expression Analysis

Western blotting: whole cell lysates (20  $\mu\text{g}$ /well) were separated by 7.5% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Run settings: 1 hour, 100V, 0.5 A) and transferred to a poly(vinylidene difluoride) membrane (PVDF) using a Criterion™ Blotter (Bio-Rad, UK) (Run settings: 30 minutes, 100 V, 0.5 A). Membranes were blocked by incubation with 5% non-fat milk (Fluka, UK) in Tris-buffered 0.1% Tween-20 (TBS-T, Bio-Rad, UK) for 1 hour at RT, cut into two sections and incubated accordingly with mouse anti-human CD44 (Clone 156-3C11, #170-5061; Cell Signaling Technology, UK) (dilution 1:1000 in TBS-T) at 4°C overnight, or rabbit anti-human  $\beta$ -actin (ab8227, Abcam, UK) (1:5000 dilution in TBS-T) for 1 hour at RT. After several washings, membranes were incubated with goat anti-mouse IgG-peroxidase (A0168, Sigma-Aldrich, UK) or goat anti-rabbit IgG-peroxidase (A0545, Sigma-Aldrich, UK), respectively, for 1 hour at RT. Bands were detected using Clarity Western enhanced chemiluminescence (ECL) substrate (Bio-Rad, UK) with the aid of a ChemiDoc™ MP System (Bio-Rad, UK). ImageJ software (<http://rsb.info.nih.gov/ij>) was used to perform a densitometric analysis of protein bands.

Flow cytometry: cells were detached using Enzyme-Free Buffer formulated in PBS (Gibco®/Invitrogen, UK).  $0.5 \times 10^6$  cells per sample tube were incubated with Fc-receptor blocking inhibitor (Affymetrix/eBioscience, UK) followed by staining with anti-human CD44 APC-conjugated (ab81424, clone MEM-263; Abcam, UK) for 30 minutes at RT. Excess unbound antibody was washed off and live analysis was performed on 10,000 individual cells with a CyAn™ ADP analyser and the Summit (v4.3) software (Beckman Coulter, Inc.).

## 2.3 Preparation of HA-coated Nanoparticles

Middle viscosity chitosan (CS) obtained from crab shells, viscosimetric average molecular weight of 656 kDa, was purchased from Sigma Aldrich (#51009219, Lot: WE44069811; Basingstoke, UK), whereas that of 35 kDa was prepared experimentally via nitrite-mediated degradation. Hyaluronic acid (HA) with weight average molecular weight of 305 kDa (GPC with static light

scattering detector, experimental) was purchased from Novozymes Biopharma A/S (Bagsvaerd, Denmark) and fluorescently-labelled (FA-HA) with fluoresceinamine (Sigma-Aldrich, UK).

Nanoparticles were synthesised as described by Almalik et al. [5]. Briefly, a 0.069 wt% CS solution and a 3  $\text{mg} \cdot \text{mL}^{-1}$  HA solution were prepared by dissolving dry purified polymer in 4.6 mM HCl or deionised water, respectively. The pH was adjusted to 5 by addition of 0.1 M NaOH in the case of the CS solution, or 0.1 M HCl in the case of the HA solution. The solutions were then filtered using either a 0.22  $\mu\text{m}$  (HA) or a 0.45  $\mu\text{m}$  (CS) filter. One-stage preparation foresees the dilution 1:2 of each polymer solution in pH 5 deionised water, and complexation by addition of a volume of the CS solution into an equal volume of the HA solution under magnetic stirring (~1000 rpm) for 30 minutes at RT.

## 2.4 Quantification of Cellular Uptake (Fluorimetry)

Cells were incubated with different concentrations of FA-HA nanoparticles using either low (HA305//CS35) or high (HA305//CS656) molecular weight chitosan for the formulation. After specified incubation times (0, 1, 2 and 4 h), cells were washed three times with PBS and lysed with RIPA buffer. The amount of membrane-bound and internalised nanoparticles was estimated on the basis of the fluorescence of the cell lysates ( $\lambda_{\text{ex}} = 485 \text{ nm}$ ;  $\lambda_{\text{em}} = 528 \text{ nm}$ ; Sensitivity: 75; Optical position: Top 50%; Light source: Xenon flash). Nanoparticles were dispersed in cell lysates to provide a calibration for the fluorescence data. The amount of membrane-bound and internalised nanoparticles was normalised by the total protein content, which was quantified using the BCA assay kit (Sigma-Aldrich, UK).

## 3 RESULTS

We have established protocols for the differentiation of THP-1 premonocytes into non-polarised (M0) macrophages, as well as for their activation via the classical (M1) or alternative (M2a) pathways (Figure 1). The efficiency of the protocols was assessed using CD14, CD11c and CD44 as differentiation markers, and CD206 and the production of TNF- $\alpha$  and IL-1 $\beta$  as M2a and M1 activation markers, respectively (data not showed).

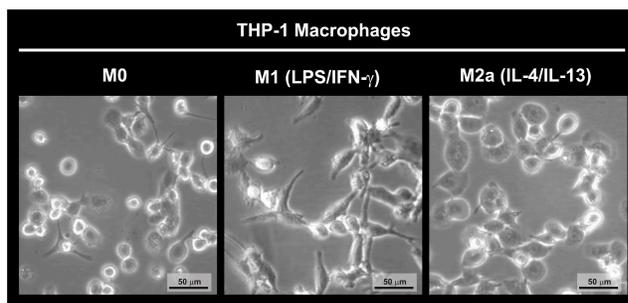


Figure 1. Morphology of THP-1 macrophages. THP-1 premonocytes were incubated with PMA to induce differentiation into non-polarised macrophages (M0). These

macrophages undergo classical (M1) or alternative (M2a) activation upon treatment with LPS/IFN- $\gamma$  or IL-4/IL-13, respectively. Representative Phase Contrast images, 40X magnification.

The expression of CD44 increased after differentiation of THP-1 monocytes into M0 macrophages; flow cytometry indicated a 1.34-fold increase, Western blotting a 0.75-fold increase. Classical activation (M1) showed further upregulation of the receptor, whereas alternative activation (M2a) appeared to have a negligible effect. In all cases we were able to detect only the standard isoform, whereas the colorectal cancer cell line HT-29 mainly expresses high molecular weight isoforms (Figure 2).

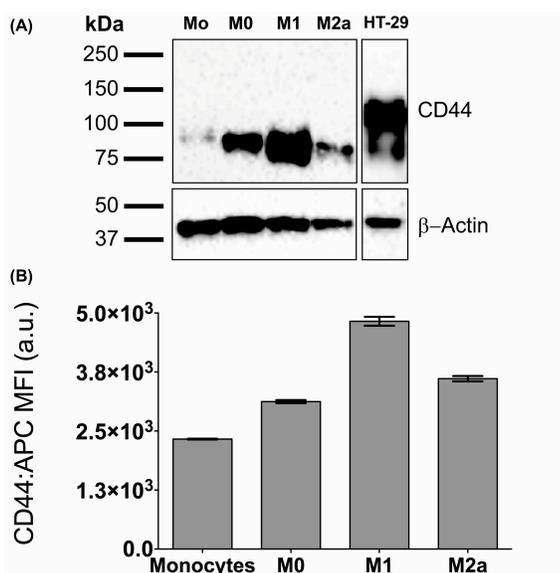


Figure 2. CD44 expression analysis. (A) Western blot showing differences in terms of expression and isoforms between THP-1 premonocytes (Mo), non-polarised THP-1 macrophages (M0), or THP-1 macrophages activated via the classical (M1) or alternative (M2a) pathways, and the HT-29 cell line.  $\beta$ -actin was used as loading control. (B) Flow cytometry analysis showing the CD44:APC median fluorescence intensity (MFI) for THP-1 premonocytes and THP-1 macrophages (M0, M1 and M2a).

Finally, we present a comparison of the kinetics of internalisation of HA-coated CS nanoparticles (Figure 3). As showed in previous publications from our group, the molecular weight of chitosan proves essential for the conformation of the HA on the surface of the nanoparticles, therefore for its presentation to CD44 on cell membranes. High molecular weight chitosan nanoparticles (HA305//CS656) were uptaken in larger amounts than their low molecular weight counterparts (HA305//CS35) for both cell models. The amount of nanoparticles internalised by THP-1 M0 macrophages was determined to be about 10% of that observed in the target HT-29 cancer cell model. This may be ascribed to a different affinity of the standard and

the variant isoforms towards HA or to a different density of CD44 on cell membranes, or both.

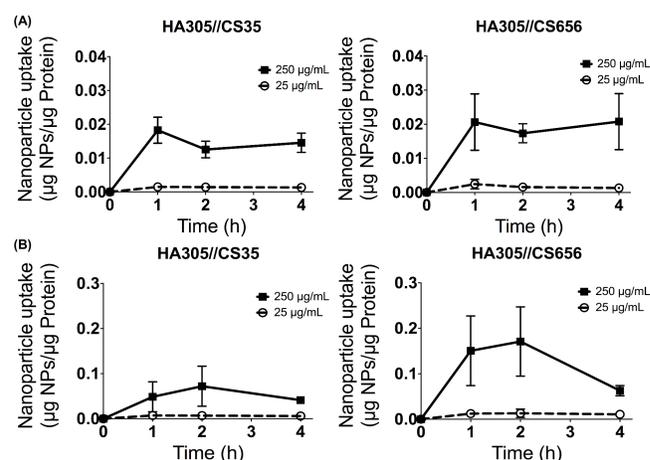


Figure 3. Uptake of HA//CS nanoparticles by (A) THP-1 M0 macrophages (B) HT-29 cells as a function of time, chitosan molecular weight and concentration of nanoparticles. The uptake was measured as the fluorescence of cell lysates (using a calibration of nanoparticles in cell lysates) and normalised against the amount of total protein content.

## 4 CONCLUSION

The THP-1 cell line provides a valuable human macrophage model to evaluate the interaction of HA-based structures with CD44. Our results suggest that the uptake of HA//CS NPs by THP-1 M0 macrophages is roughly 10% of that seen in the cancer cell line HT-29. The complete validation of THP-1 cells as an off-target model, however, would still require a comparison to primary and/or *in vivo* models.

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## REFERENCES

- Zoller, M., *CD44: can a cancer-initiating cell profit from an abundantly expressed molecule?* Nature Reviews Cancer, 2011. **11**(4): p. 254-267.
- Williams, K., et al., *CD44 integrates signaling in normal stem cell, cancer stem cell and (pre)metastatic niches.* Experimental Biology and Medicine, 2013. **238**(3): p. 324-338.
- Vivers, S., I. Dransfield, and S.P. Hart, *Role of macrophage CD44 in the disposal of inflammatory*

- cell corpses*. *Clinical Science*, 2002. **103**(5): p. 441-449.
4. Vachon, E., et al., *CD44 is a phagocytic receptor*. *Blood*, 2006. **107**(10): p. 4149-4158.
  5. Almalik, A., P.J. Day, and N. Tirelli, *HA-Coated Chitosan Nanoparticles for CD44-Mediated Nucleic Acid Delivery*. *Macromolecular Bioscience*, 2013. **13**(12): p. 1671-1680.
  6. Almalik, A., et al., *Hyaluronic acid (HA) presentation as a tool to modulate and control the receptor-mediated uptake of HA-coated nanoparticles*. *Biomaterials*, 2013. **34**(21): p. 5369-5380.
  7. Auwerx, J., *The Human Leukemia-Cell Line, Thp-1 - a Multifaceted Model for the Study of Monocyte-Macrophage Differentiation*. *Experientia*, 1991. **47**(1): p. 22-31.
  8. Tsuchiya, S., et al., *Induction of Maturation in Cultured Human Monocytic Leukemia-Cells by a Phorbol Diester*. *Cancer Research*, 1982. **42**(4): p. 1530-1536.
  9. Daigneault, M., et al., *The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte-Derived Macrophages*. *Plos One*, 2010. **5**(1).