Mimicking interactions between colorectal cancer cells and tumor-associated macrophages using Nunc cell culture inserts in a carrier plate system

Abstract

Co-culture systems are widely used to investigate interactions between different cell types and often require specialized culture vessels to model them *in vitro*. This study utilized **Thermo Scientific™ Nunc™ cell culture inserts in a carrier plate system** to demonstrate the effects of tumor-associated macrophages (TAMs) on the behavior of colorectal cancer (CRC) cell line HCT116. The TAMs were co-cultured with HCT116 cells, and changes in their migration, invasive properties, and expression of epithelial-mesenchymal transition (EMT)-related genes were demonstrated.

Introduction

Cell culture is a process of growing cells in a laboratory, wherein cells reproduce and survive under controlled, artificial conditions. For mammalian cells, the cells are typically grown on treated surfaces as a single monolayer culture consisting of a single cell type. In contrast, co-culture is an *in vitro* method in which two or more types of cells are cultured together with some degree of contact between them. This can be direct physical contact, or indirect contact via cytokines and growth factors. Such interactions influence the survival, differentiation, and maturation of cells, and co-culture helps mimic the multicellular organization and complexity of the *in vivo* microenvironment [1-5]. Co-culture also helps in evaluating cross-communication between cells [6-8] and is commonly used for drug development [9], tissue engineering [10], and studying the initiation, promotion, and progression of various diseases [11].

Here we describe an indirect co-culture model for TAMs and CRC cell line HCT116 that was established using the Thermo Scientific™ Nunc™ carrier plate system with Nunc™ cell culture inserts. Each system consists of three parts: a multi-well cell culture plate, a carrier plate containing Nunc cell culture inserts with porous membranes, and a lid. Nunc cell culture inserts are designed to maintain cells in two different layers, enabling the study of specific cell functions. This study utilized the Nunc carrier plate system with cell culture inserts to highlight the effects of TAMs on the behavior of CRC cells in the context of migration, invasion, and EMT.

CRC is the second most prevalent cause of cancer death around the world [12]. Understanding crosstalk between CRC cells and factors like TAMs will contribute to a better understanding of the cancer microenvironment and may help identify new therapeutic strategies. TAMs are a mature population of terminally differentiated myeloid-lineage cells present in or proximal to primary tumors [13]. TAMs play an important role in processes like tumor progression, invasion, angiogenesis, and metastasis [14,15]. However, their role in CRC progression is currently not clear [16]. While some studies report that TAMs improve survival and reduce metastasis [17,18], others suggest that TAM infiltration is associated with advanced tumor stages, poor prognosis, and short survival [19-21]. Therefore, continual investigation on the role of TAMs in CRC progression would help improve therapeutic strategies.

In this study, $CD163^+$ TAMs were generated *in vitro* and co-cultured with CRC cell line HCT116 using the Nunc carrier plate system with cell culture inserts, which led to increased migration and invasion of the cancer cells. Because TAMs have been shown to play a critical role in tumor metastasis by regulating EMT [22-25], we tested the ability of TAMs to induce changes in the expression of EMT-related genes in HCT116 cells, using customized **Applied Biosystems™ TaqMan® Array Plates**. This study successfully showed interaction between TAMs and CRC cells and demonstrated the versatility of the Nunc carrier plate system with cell culture inserts for use in a wide range of co-culture applications.

Materials

Refer to the ordering table on the last page for details about the materials used.

Reagent preparation

Geltrex matrix solution

Gibco™ Geltrex™ LDEV-Free Matrix was thawed overnight at 4°C, and 1 part Geltrex matrix was diluted with 4 parts cold 1X PBS.

Formaldehyde (FA) solution

16% FA was diluted to 4% using 1X PBS.

Caution: FA is toxic and corrosive; handle in a fume hood and avoid any direct contact.

Crystal violet solution

1% crystal violet stain was diluted to 0.2% using 1X PBS.

Methods

Cell culture

Human monocyte leukemia cell line THP-1 was cultured in Gibco™ RPMI 1640 Medium with 10% FBS. **CRC cell line HCT116** was cultured in DMEM supplemented with 10% FBS. The normal fetal human colon (FHC) epithelial cell line was grown in **Gibco™ DMEM/F-12** with 10% FBS, 10 mM HEPES, 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone, and 20 ng/mL human recombinant EGF per vendor guidelines. All cells were grown at 37°C in a humidified atmosphere with 5% CO_2 following vendor recommendations.

Generation of TAMs

THP-1 cells at a density of 0.2 x 10^6 cells/mL were treated with 200 nM phorbol 12-myristate 13-acetate (PMA) for 24 hr. The polarized THP-1-PMA macrophages were then treated with conditioned medium from the HCT116 cells for another 48 hr to obtain TAMs. To generate the conditioned medium, HCT116 cells were seeded at a density of 50,000 cells/cm^2 and grown for 96 hr in DMEM with 10% FBS. After 96 hr, spent medium from these cells was used as conditioned medium. TAM generation was validated based on morphology using microscopy and analysis of surface marker expression by flow cytometry. Conditioned medium from the FHC cell line was used as a negative control; spent medium from FHC cells seeded at a density of 10,000 cells/cm^2 and grown for 96 hr was added to THP-1-PMA macrophages, and their morphology and surface marker expression were compared with those of the TAMs. CM from FHCs added to THP-1-PMA macrophages should not induce TAM formation.

Flow cytometry

Following 48 hr of exposure to medium conditioned to normal (FHC) or CRC (HCT116) cells, CD163 expression was assessed by flow cytometry. Cells were suspended in 1X PBS at 1 x 10^6 cells/mL, from which 100 µL was taken and incubated with anti-CD163 antibody for 30 min at 4°C. The cells were then washed twice with 100 µL 1X PBS and resuspended in 0.3 mL of focusing fluid for analysis. Flow cytometry was performed using an Invitrogen™ Attune™ NxT Flow Cytometer.

Wound healing assay

HCT116 cells were seeded into the receiver wells of a 24-well cell culture plate at a density of 1 x 10^5 cells/cm^2. Once the cells were 80–90% confluent, a wound was made using a 10 µL pipette tip. The wells were washed with PBS to remove cellular debris, and 500 µL of serum-free medium was added. Nunc carrier plates with height adjustment grooves were used to hold Nunc cell culture inserts with a 0.4 µm pore size that contained TAMs or THP-1-PMA macrophages. The cell culture inserts were positioned at medium height and placed on top of the wells containing wounded HCT116 cells, then incubated at 37°C for 24 hr. Wounded HCT116 cells without cell culture inserts were included as a negative control. After 24 hr, migrated cells were photographed using the **Invitrogen™ EVOS™ M7000 Imaging System**, and the wounded area was analyzed in polygon selection mode using ImageJ software. All experiments were performed in duplicate.

Crystal violet staining for the invasion assay

To establish an invasion assay, **24-well Nunc cell culture inserts** with 8 µm pores were first coated with Geltrex matrix. The Geltrex matrix was diluted as described in the reagent preparation section above, and 100 µL of the diluted matrix was added to the inserts and kept at 37°C for 1 hr. Once the Geltrex matrix solidified, 1 x 10^5 HCT116 cells were suspended in 200 µL of DMEM with 1% FBS and added to the Nunc cell culture inserts. The TAMs and THP-1-PMA macrophages were placed in the receiver wells of a 24-well cell culture plate. A chemoattractant, DMEM supplemented with 10% FBS, was placed in the receiver well to serve as a positive control. After 24 hr of incubation, the cells remaining in the upper chamber were removed using cotton swabs. Cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. Cells were imaged in multiple microscopic fields at 10x magnification. All experiments were performed in duplicate.

Co-culture for EMT gene expression

HCT116 cells were seeded in the receiver wells of a 24-well cell culture plate at a density of 5 x 10^4 cells/cm^2. THP-1 cells were added at a density of 5 x 10^4 cells/cm^2 to the upper chamber of an 8 µm pore size Nunc cell culture insert and polarized into TAMs or THP-1-PMA macrophages (Figure 1). The cells were then incubated in a humidified atmosphere containing 5% CO_2 at 37°C. After 48 hr of co-culture incubation, HCT116 cells were harvested for qPCR analysis.

RT-qPCR

Total RNA was isolated from HCT116 cells using the Invitrogen™ TRIzol™ Plus RNA Purification Kit following the manufacturer's instructions. The RNA (1 µg) was then reverse-transcribed to cDNA using a high-capacity cDNA reverse transcription kit. EMT gene expression was analyzed using a customized EMT TaqMan Array Plate. The PCR conditions were as follows: 40 cycles of denaturation at 95°C for 20 sec; annealing at 95°C for 3 sec; and extension at 60°C for 20 sec. The mRNA level for each gene was normalized to that of β-actin. Expression of target proteins was calculated using the $2^{-\Delta\Delta}C_t$ method [26].

Generation of TAMs

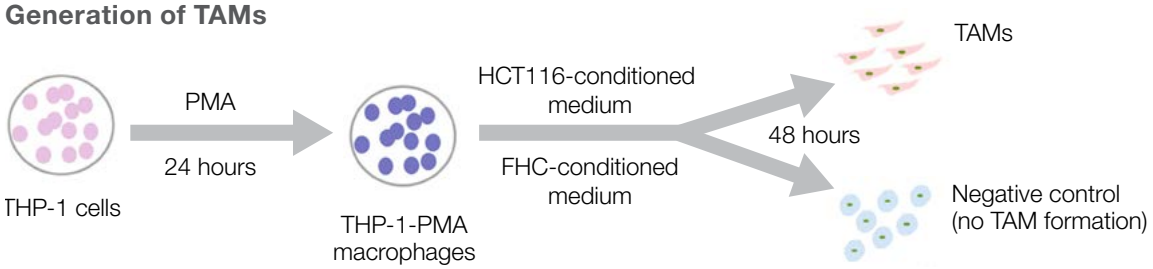

Figure 1. Experimental procedure used to generate TAMs.

Results

Generation and validation of TAMs

An *in vitro* model for TAMs was generated by treating THP-1 cells with PMA and HCT116-conditioned medium (Figures 1, 2). This was validated based on cellular morphology and surface marker expression. As shown in Figure 2A, when the conditioned medium from HCT116 was added to PMA-treated THP-1 cells, they became elongated. By flow cytometry, these cells also showed higher expression of CD163 (Figure 2B). Conditioned medium from the FHC cells was used as a negative control, and addition of the conditioned medium did not change the morphology of the PMA-treated THP-1 cells or the expression of surface markers (Figure 2B).

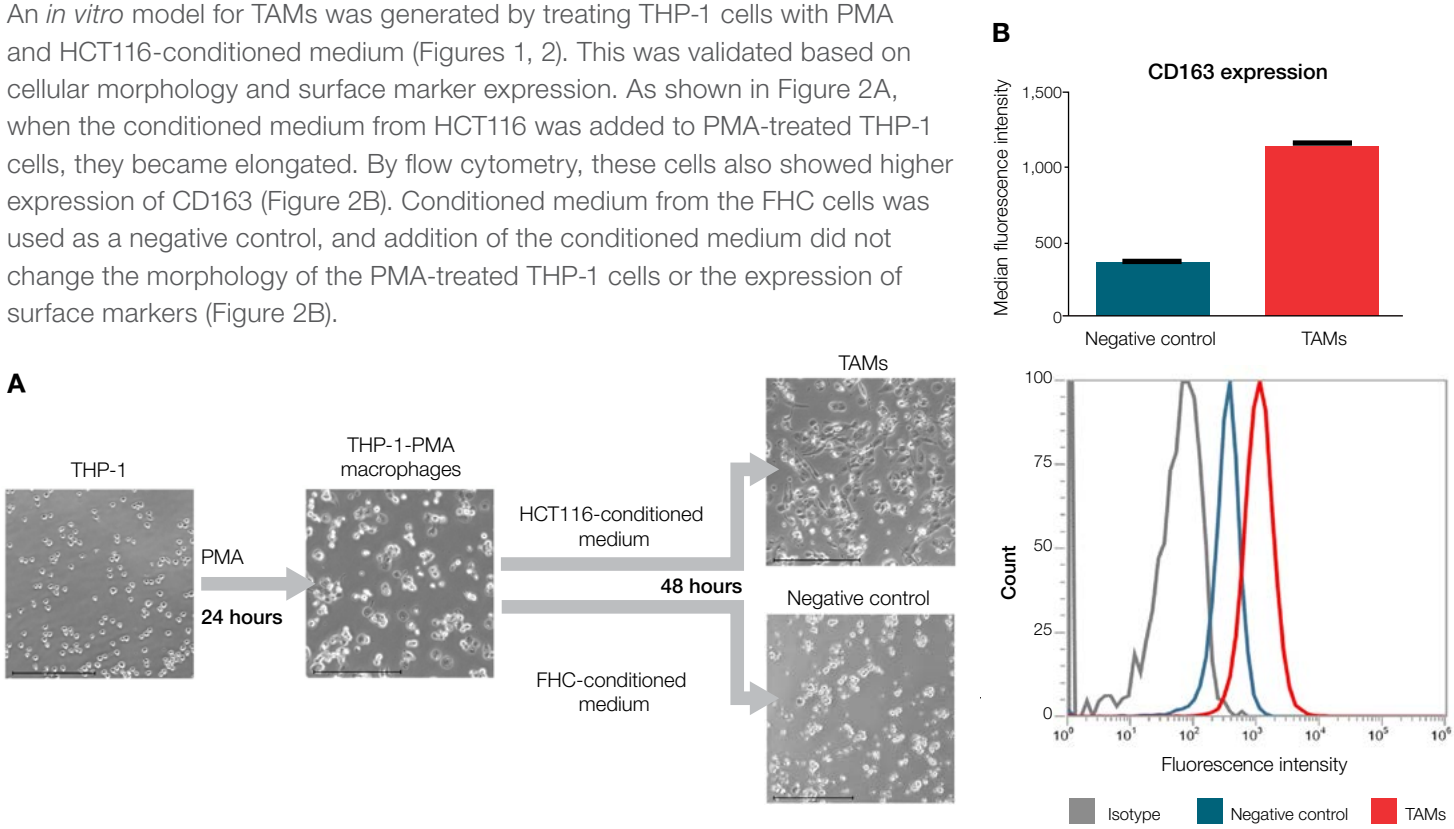

Figure 2. TAM morphology and surface marker expression. (A) THP-1 cells treated with PMA for 24 hr were cultured with HCT116- or FHC-conditioned medium for 48 hr. The representative bright-field images were captured using an EVOS M7000 microscope at 10x magnification (scale bar represents 275 µm). **(B)** Flow cytometry analysis showed that THP-1-PMA macrophages incubated with HCT116-conditioned medium expressed more CD163 than those incubated with FHC-conditioned medium.

Wound healing

To determine their impact on the migratory capacity of HCT116 cells, TAMs and THP-1-PMA macrophages were co-cultured with HCT116 cells using Nunc cell culture inserts with 0.4 µm pores. This pore size allowed the exchange of secreted factors, but the inserts were impermeable to the cells. Cell migration was measured by determining the movement of cells into a scratched area mimicking a wound, which was created with a pipette tip. Images were captured immediately after the wounds were created to record the initial area of the wounds. The wound locations were imaged after 24 hr to evaluate the recovery of the monolayers due to cell migration, which was expressed as a percentage of wound closure. As seen in Figure 3B, co-culture with TAMs resulted in significantly faster wound closure over 24 hr.

Percentage of wound closure:

$$\frac{\text{(Area of wound measured immediately after scratching (0 hr)) – (Area of the wound measured 24 hr after scratching)}}{\text{(Area of wound measured immediately after scratching (0 hr))}} \times 100$$

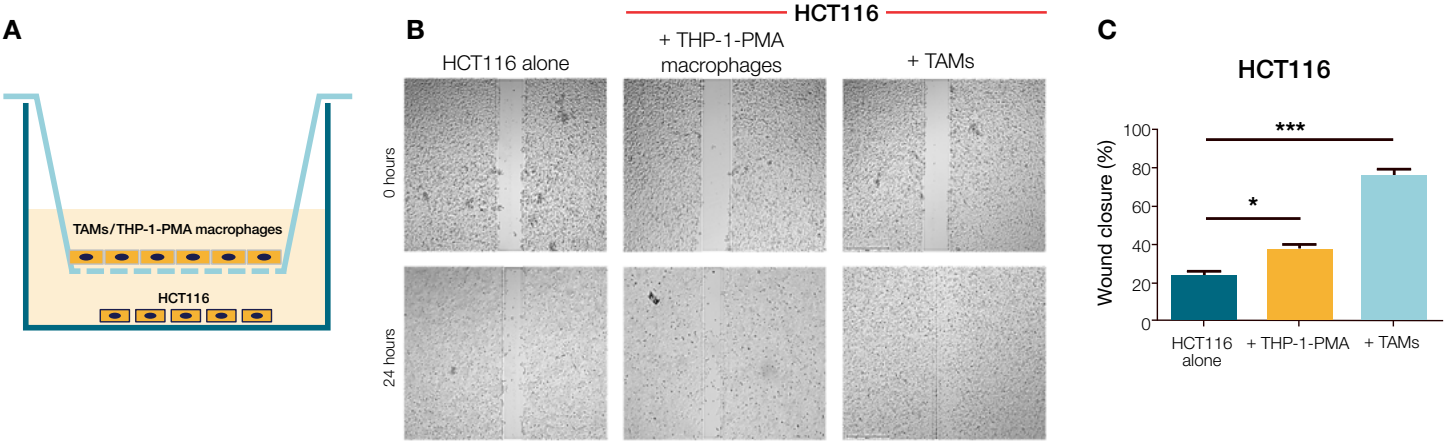

Figure 3. Visualization and determination of wound healing. (A) Experimental setup to evaluate wound healing. A similar setup was used for control conditions. **(B)** Representative bright-field images of wound healing to determine the migration capacity of HCT116 cells co-cultured with PMA-treated THP-1 macrophages and TAMs. Images were captured at 10x magnification using an EVOS M7000 microscope. **(C)** The extent of wound closure was calculated by analyzing the scraped area covered by migrated cells after 24 hr using ImageJ software. The data are reported as the mean ± SEM of two independent experiments (*$P < 0.01$, ***$P < 0.0001$, n = 2).

Invasion assay

Given its impact on migratory potential, co-culture with TAMs was also tested to assess the impact on the invasiveness of HCT116 cells. HCT116 cells were plated on Geltrex matrix–coated cell culture inserts in the presence of either TAMs or THP-1-PMA macrophages. After 24 hr of co-culture, cells that had migrated towards the lower chamber were stained with crystal violet. Compared to the control, HCT116 cells co-cultured with TAMs showed higher invasion through the Geltrex matrix barrier (Figure 4).

EMT gene expression

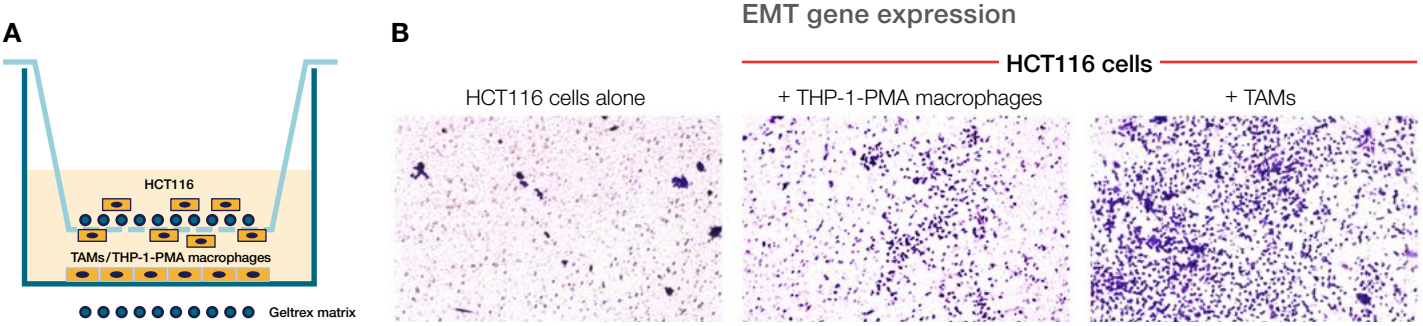

Figure 4. Schematic and visualization of an invasion assay. (A) Experimental setup for performing the invasion assay. **(B)** Representative images of HCT116 cells that migrated through Geltrex matrix–coated membranes in response to FBS (HCT116 alone, left), co-cultured with THP-1-PMA macrophages (middle), and co-cultured with TAMs (right). Images were taken at 10x magnification using an EVOS M7000 microscope.

In an effort to connect enhanced migratory and invasive potential with EMT, the expression by HCT116 cells of genes associated with EMT was analyzed using a **TaqMan array plate**. Expression of genes for the epithelial markers E-cadherin (*CDH1*) and desmoplankin (*DSP*) by HCT116 cells was reduced when they were co-cultured with TAMs, while expression of genes for the mesenchymal markers vimentin (*VIM*), fibronectin 1 (*FN1*), and N-cadherin (*CDH2*) was upregulated (Figure 5). Lower expression of *CDH1* coupled with a rise in *CDH2* and *VIM* expression is a typical molecular signature of EMT. This suggests that the HCT116 cells may have undergone molecular alterations in the presence of TAMs that could result in a transition from an epithelial phenotype to a more mesenchymal phenotype. However, we were unable to draw a definite conclusion with respect to the expression of other EMT-related genes, such as *MMP2*, *MMP9*, and *TWIST1*. Manipulating co-culturing incubation times and conditions might provide additional information about the effect of TAMs on the EMT gene expression profile of cancer cells. Further investigation may reveal important regulatory mechanisms and additional molecular changes underlying EMT.

Find out more about how our one-of-a-kind carrier plate and inserts can be height-adjusted inside each well to achieve optimal positions relative to the medium and bottom of the plate. **Learn more here.**

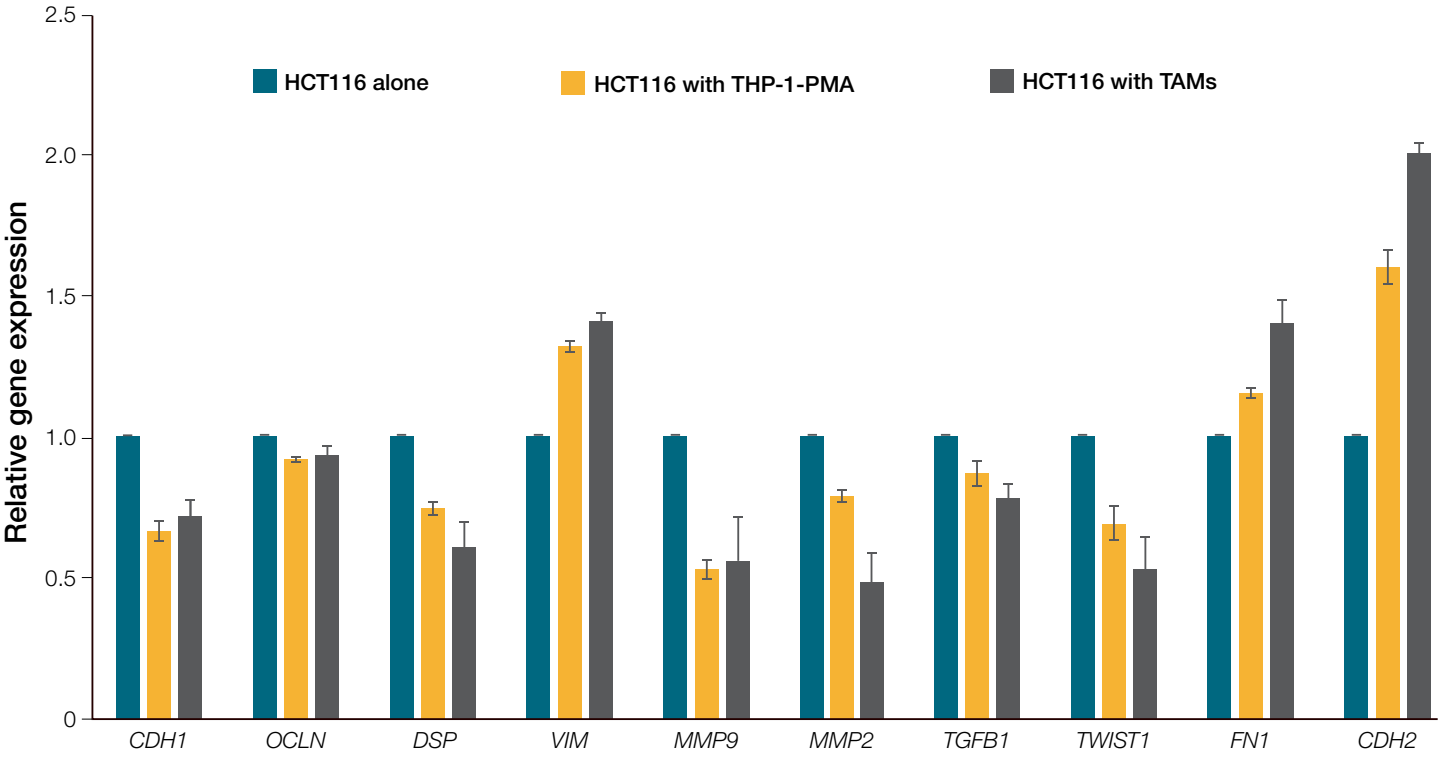

Figure 5. Expression of EMT-related gene by HCT116 cells co-cultured with TAMs or THP-1-PMA macrophages, evaluated by qPCR analysis.

Conclusions

Co-culture using **Nunc cell culture inserts in a carrier plate system** is a powerful tool for creating better models of *in vivo* environments. Combining multiple cell types in an *in vitro* model will help advance our knowledge about fundamental mechanisms in biological systems. In this study, Nunc cell culture inserts in a carrier plate system were used to co-culture HCT116 cells with TAMs to investigate wound healing, invasion, and expression levels of EMT-related genes. These Nunc cell culture inserts are easy to use and suitable for performing co-culture studies. They can be easily adapted to other applications to better understand complex biological systems.

Ordering information

Supplier	Materials	Cat. No.
	Reagents	
Thermo Fisher Scientific	**RPMI 1640 Medium**	**11875093**
	Fetal Bovine Serum	**Various**
	DMEM, high glucose, GlutaMAX Supplement	**10569-010**
	TrypLE Express Enzyme (1X), phenol red	**12605010**
	HEPES	**15630080**
	Insulin	**A11382II** (sourced from Biocon)
	Human recombinant EGF	**PHG0311**
	PBS (phosphate-buffered saline), pH 7.4	**10010049**
	Pierce 16% Formaldehyde (FA)	**28906**
	Geltrex LDEV-Free Matrix	**A1413201**
	TRIzol Plus RNA Purification Kit and Phasemaker Tubes Complete System	**A33254**
	High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor	**4374966**
	TaqMan Fast Advanced Master Mix	**4444556**
	Customized TaqMan Array Fast Plate	**4413255**
Sigma-Aldrich	Cholera toxin	C8052
	Transferrin	T8158
	Phorbol 12-myristate 13-acetate (PMA)	P1585
	Crystal violet solution, 1%	V5265
	Plastics	
Thermo Fisher Scientific	**Nunc Cell Culture Inserts in Carrier Plate, 24-well format**	**141006, 141002**
	Laboratory equipment	
Thermo Fisher Scientific	**EVOS M7000 Imaging System**	**AMF7000**
	QuantStudio 5 Real-Time PCR System	**A28138**
	Attune NxT Flow Cytometer	**A24858CFR**
	Others	
	Cotton swabs, CO_2 incubator, laminar hood, centrifuge	
	Product	
Selleckchem	Hydrocortisone	S1696

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