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1,25-dihydroxyvitamin D_3 augments low-dose PMA-based monocyte-to-macrophage differentiation in THP-1 cells

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ABSTRACT

The human monocytic THP-1 cell line is the most routinely employed *in vitro* model for studying monocyte-to-macrophage differentiation. Despite the wide use of this model, differentiation protocols using phorbol 12-myristate-13-acetate (PMA) or 1,25-dihydroxyvitamin D_3 ($1,25D_3$) vary drastically between studies. Given that differences in differentiation protocols have the potential to impact the characteristics of the macrophages produced, we aimed to assess the efficacy of three different THP-1 differentiation protocols by assessing changes in morphology and gene- and cell surface macrophage marker expression. THP-1 cells were differentiated with either 5 nM PMA, 10 nM $1,25D_3$, or a combination thereof, followed by a rest period. The results indicated that all three protocols significantly increased the expression of the macrophage markers, CD11b ($p < 0.001$) and CD14 ($p < 0.010$). Despite this, THP-1 cells exposed to $1,25D_3$ alone did not adopt the morphological and expression characteristics associated with macrophages. PMA was required to produce these characteristics, which were found to be more pronounced in the presence of $1,25D_3$. Both PMA- and PMA with $1,25D_3$-differentiated THP-1 cells were capable of M1 and M2 macrophage polarization, though the gene expression of polarization-associated markers was most pronounced in PMA with $1,25D_3$-differentiated THP-1 cells. Moreover, the combination of PMA with $1,25D_3$ appeared to support the process of commitment to a particular polarization state.

1. Introduction

Monocyte-derived macrophages are highly plastic innate immune cells that are integral in pathogen detection, phagocytosis, and immune cell recruitment (Hirayama et al., 2017), as well as maintaining tissue homeostasis and participating in tissue repair (Bassler et al., 2019). Dysregulation of the macrophage compartment has been shown to contribute to several disease pathologies, including auto-immune disorders (Ma et al., 2019), neurodegeneration (Park et al., 2022), and cancer (Boutilier and Elsawa, 2021). Following monocyte recruitment, monocytes leave the circulatory system and enter tissues, through leukocyte extravasation, and in the process of doing so, differentiate into macrophages (Rutledge and Muller, 2020; Medrano-Bosch et al., 2023).

Key functional aspects that distinguish tissue-resident macrophages from their monocyte precursors include antigen presentation (Italiani and Boraschi, 2014), enhanced phagocytosis (Uribe-Querol and Rosales, 2020), and enhanced participation in inflammatory immune responses (Bassler et al., 2019). Depending on the nature of the tissue environment, these macrophages adopt one of two broad polarization states, either becoming pro-inflammatory, antigen presenting M1 macrophages, or highly phagocytic, anti-inflammatory M2 macrophages (Shapouri-Moghaddam et al., 2018; Yao et al., 2019). In practice, the differential expression of integrins, cell adhesion molecules, phagocytic receptors, cytokines, major histocompatibility complex class I and class II molecules, and specific transcription factors are frequently employed to distinguish monocytes from macrophages (Hesketh et al., 2017; Baxter et al., 2020) and to define macrophage polarization states (Hesketh et al., 2017; Chistiakov et al., 2018; Tedesco et al., 2018; Orekhov et al., 2019; Mohd Yasin et al., 2022; Rynikova et al., 2023).

As the use of patient samples or animal models is not always feasible or practical, the use of a reliable cell line model for investigations into monocyte-to-macrophage differentiation, macrophage polarization, and the relative contribution of these cells to health and disease is necessary. THP-1 cells (Tsuchiya et al., 1980) can be differentiated from a monocyte-like state to a more macrophage-like state using differentiation agents such as phorbol 12-myristate 13-acetate (PMA) and 1,25-dihydroxyvitamin D_3 ($1,25D_3$) - the biologically active form of vitamin D_3 (Daigneault et al., 2010; Rynikova et al., 2023). However, despite the

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widespread use of this model, there is limited consensus on the differentiation protocol that should be employed. This is important as the differentiation protocol used is known to affect the resulting phenotype of the macrophages produced and alter the response of these cells to various stimuli (Park et al., 2007; Maeß et al., 2014; Lund et al., 2016; Rynikova et al., 2023).

Notably, differentiation using high concentrations of PMA has been shown to inhibit the response to LPS stimulation (Park et al., 2007; Lund et al., 2016) and prevent effective M2 macrophage polarization (Maeß et al., 2014). This can be avoided by either reducing the concentration of PMA used for differentiation or using an alternative differentiation agent. However, the use of low PMA concentrations results in a phenomenon referred to as de-differentiation, wherein the cells regain proliferative capacities and a monocyte-like phenotype (Spano et al., 2013). Similarly, the use of alternative differentiation agents, including $1,25D_3$, may fail to produce a macrophage-like phenotype (Daigneault et al., 2010; Rynikova et al., 2023). This failure to differentiate may be overcome by combining low PMA concentrations together with $1,25D_3$, as has been suggested for the U937 monocyte-like cell line (Valdés López and Urcuqui-Inchima, 2018). In this study, we therefore explored three potential THP-1 differentiation protocols, using either 5 nM PMA, 10 nM $1,25D_3$, or a combination thereof. We found that while $1,25D_3$ alone failed to induce the differentiation of THP-1 cells to a more macrophage-like state, this was achievable with low concentrations of PMA. However, combining low-dose PMA with $1,25D_3$ further augmented the macrophage-like characteristics in THP-1 cells. Finally, we demonstrate that macrophages differentiated using PMA together with $1,25D_3$ can be successfully polarized into distinct M1- and M2- states, characterized by the expression of CD80 and CD206, respectively.

2. Materials and methods

2.1. Cell culture and differentiation

The THP-1 cell line (HIV Reagent program ARP-9942) was a generous gift from Dr. H. Ranchod (NICD, South Africa). THP-1 cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine (Pan Biotech, Aidenbach, German), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS; Pan Biotech, Aidenbach, Germany) and incubated at 37 °C with 5% CO_2. Cells were routinely tested for, and found to be free of, mycoplasma contamination using the MycoStrip mycoplasma detection kit (Invivogen, San Diego, CA, USA). THP-1 cells (0.5×10^6 cells/ml; passage 5–10) were differentiated in the presence of 10 nM $1,25D_3$ for 96 h, or in the presence of 5 nM PMA for 24 h, followed by a 72-h rest period in culture media, or in the presence of 5 nM PMA with 10 nM $1,25D_3$ for 24 h, followed by a 72-h rest period in culture media supplemented with 10 nM $1,25D_3$. A 72-h rest period was included as it enhanced macrophage morphology (data not shown). A concentration of 5 nM PMA was selected as it was the lowest PMA concentration capable of inducing cell adherence, while maintaining cell viability (Fig. S1). THP-1 cells exposed to EtOH, DMSO, or a combination thereof, were used as vehicle controls for the 10 nM $1,25D_3$, 5 nM PMA, and 5 nM PMA with 10 nM $1,25D_3$ conditions, respectively. Following 96 h of differentiation, adherent cells were detached from the plate using 0.25% Trypsin-EDTA (Sigma Aldrich, St. Louis, MO, USA). Cells were washed with phosphate buffered saline (PBS; pH 7.4 Sigma Aldrich, St. Louis, MO, USA), counted, and assessed for viability using the Trypan-blue exclusion assay (viability >94% in all cases, Fig. S2). Cell pellets were stored in 200 µl RNA*later* (Thermofisher Scientific, Waltham, MA, USA) at −80 °C for RNA extractions (1×10^6 cells) or used immediately for flow cytometry (2×10^6 cells).

2.2. Transmitted light microscopy

Morphological alterations associated with the process of monocyte-to-macrophage differentiation, such as an increase in cell size and adherence, were captured by an EVOS FLoid imaging system (Thermofisher Scientific, Waltham, MA, USA) with a fixed 20× objective lens (460× magnification). Representative images presented are reflective of at least five images.

2.3. Macrophage surface marker quantification

Increases in cell size, cellular complexity, and macrophage surface marker expression were assessed by flow cytometry. Flow cytometric analysis was conducted using a BD Accuri C6 flow cytometer (BD Accuri, Ann Arbor, MI, USA). For each differentiation condition evaluated, three biological replicates and two technical replicates of 1×10^6 cells were included. Following harvesting, cells were resuspended in 50 µl of 10% (*v*/v) heat-inactivated human serum for 1 min. Once the human serum was removed, the cells were fixed by resuspending the cell pellet in 50 µl of 1.5% (*w/v*) paraformaldehyde (Sigma Aldrich, St. Louis, MO, USA) in PBS for 30 min. The fixed cells were then stained simultaneously with 5 µl of FITC conjugated mouse IgG_1 anti-human CD11b clone ICRF44 (0.5 µg/ test) and APC conjugated mouse IgG_1 anti-human CD14 clone 61D3 (0.25 µg/ test) monoclonal antibodies (Invitrogen, Waltham, MA, USA) suspended in 40 µl of 1% (w/v) bovine serum albumin (BSA; Roche, Basel, Switzerland) in PBS and incubated for 30 min. The antibody solution was removed, and the cell pellets were washed twice in 0.1% (v/v) Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) in PBS by centrifugation at 800 ×*g* for 8 min. Washed cells were resuspended in a final volume of 150 µl 1% (w/v) BSA in PBS. For each replicate, a total of 75,000 events were captured.

2.4. RNA sequencing

RNA extraction, library preparation, and mRNA sequencing services for three biological replicates of the 5 nM PMA, and 5 nM PMA with 10 nM $1,25D_3$ differentiation conditions and their matching vehicle controls were provided by Admera Health, LLC (South Plainfield, NJ). RNA extractions were performed using the RNeasy Plus 96 kit (Qiagen, Valencia, CA), according to the manufacturer's specifications. RNA quantity was assessed using the Qubit RNA HS assay (Thermofisher Scientific, Waltham, MA, USA), while quality was assessed using the Bioanalyzer 2100 Eukaryote Total RNA Nano (Agilent Technologies, CA, USA). All replicates were found to have RIN values ≥9.8. Paired-end sequencing libraries were constructed using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA), in accordance with manufacturer's guidelines. Sequencing was performed on the NovaSeq 6000 S4, with a minimum of 20 million reads (2×150 bp) obtained for each replicate. The sequencing data generated during this study have been submitted to the European Nucleotide Archive (ENA) and are available under the study accession PRJEB76891.

2.5. Differential gene expression analysis

Quality control of the raw sequencing data was done using FastQC v0.11.9 (Andrews, 2010). Estimation of transcript abundances was performed using Salmon v1.10.0 (Patro et al., 2017), against the GENCODE v43 annotations of GRCh38, and transcript-level expression estimates were collapsed to the gene level using tximport v1.26.1 (Soneson et al., 2015). Differential gene expression analysis was conducted using DESeq2 v1.39.3 (Love et al., 2014), incorporating $\log_2$ fold change shrinkage estimates derived from apeglm v1.22.1 (Zhu et al., 2019), where each differentiation condition was compared to its corresponding vehicle control. Genes with low counts (DESeq2 base mean < 20) were removed prior to significance testing, and genes were

considered to be significantly differentially expressed if they had an absolute $\log_2$ fold change >1 and a Benjamini-Hochberg adjusted *p*-value <0.05 (DEGs). RT-qPCR validation of the results obtained from differential gene expression analysis was performed for C-X-C motif chemokine ligand 10 (*CXCL10*; Fig. S2).

2.6. Macrophage polarization

Following differentiation, 5 nM PMA and 5 nM PMA with 10 nM 1,25D_3 differentiated THP-1 derived macrophages were polarized to an M1 state by exposure to 100 ng/ml lipopolysaccharide (LPS) and 20 ng/ml interferon γ (IFN-γ) over 24 h. M2 macrophage polarization was achieved with 20 ng/ml interleukin 4 (IL-4) and 20 ng/ml interleukin 13 (IL-13) over 48 h (Wei and Besner, 2015; Zhang et al., 2019). Accompanying alterations in morphology were assessed by microscopy. The M1- and M2- specific macrophage polarization markers, CD80 and CD206 (encoded by *MRC1*), were quantified by flow cytometry with 5 μl of APC conjugated mouse $IgG_{2\alpha,\kappa}$ anti-human CD80 clone W17149D and FITC conjugated mouse $IgG_{1\kappa}$ anti-human CD206 clone 15–2 monoclonal antibodies (Biolegend, San Diego, CA, USA), respectively.

2.7. Statistical analysis

To determine significant alterations in FSC and SSC and the expression of CD11b, CD14, CD80 and CD206, a one-way analysis of variance (ANOVA) was conducted, followed by a Fisher's least significant difference (LSD) test. Statistical analyses were performed using IBM SPSS v. 28.0.1.0(142).

3. Results

3.1. PMA is required to produce the morphological characteristics associated with macrophage differentiation in THP-1 cells

Transmitted light microscopy was used to assess changes in morphology in THP-1 cells differentiated with 5 nM PMA, 10 nM 1,25D_3, and 5 nM PMA with 10 nM 1,25D_3. 1,25D_3-differentiated THP-1 cells did not demonstrate an increase in cell size, or any alteration in cell shape (Fig. 1B). While a moderate degree of cellular adherence was observed, it was not firm adherence, and was not accompanied by cell flattening. For cells to achieve the characteristic, prototypical macrophage morphology, including firm cellular adherence, cell flattening, alterations in cell shape, and an increase in cell size, PMA was required. These characteristics were observed in PMA- and PMA with 1,25D_3-differentiated THP-1 cells, albeit to different extents (Fig. 1C-D). In contrast to when treated with PMA alone, more cells adopted a macrophage-like morphology when exposed to PMA with 1,25D_3. The PMA-differentiated THP-1 cells were predominantly large and rounded, with a few spindle-like cells, while the PMA with 1,25D_3-differentiated THP-1 cells were larger and predominantly elongated, spindle-like cells. Flow cytometry further confirmed these observations, with PMA- and PMA with 1,25D3-differentiated THP-1 cells demonstrating a significant increase in cell size (FSC; $p < 0.001$) and granularity (SSC; PMA: $p < 0.010$, PMA with 1,25D3: $p < 0.001$), as opposed to 1,25D_3-differentiated THP-1 cells, which showed no significant increase in either FSC or SSC (Fig. 2A-B).

3.2. Both PMA and 1,25D_3 contribute to an increase in CD11b and CD14 protein expression in THP-1 macrophages

Flow cytometry was used to quantify changes in the expression of the macrophage cell surface markers, CD11b and CD14 (Park et al., 2007; Aldo et al., 2013; Spano et al., 2013; Starr et al., 2018) in response to differentiation. A significant increase in the expression of CD11b ($p < 0.001$; Fig. 3A) and CD14 ($p < 0.010$; Fig. 3B), relative to control cells, was observed in all the differentiation conditions assessed. However, the expression of CD11b and CD14 was not significantly different between 1,25D_3_or PMA-differentiated THP-1 cells. PMA with 1,25D_3-differentiated THP-1 cells showed significantly higher CD11b ($p < 0.001$) and CD14 expression ($p < 0.001$) when compared to the other differentiation conditions employed. This suggested that although exposure to 1,25D_3 alone did not produce the other morphological characteristics associated with monocyte-to-macrophage differentiation, it did result in the increased expression of CD11b and CD14.

3.3. 1,25D_3 increases the expression of traditional and primary macrophage markers in PMA differentiated THP-1 cells

Given that 1,25D_3 alone failed to induce the morphological features associated with successful macrophage differentiation, differential gene

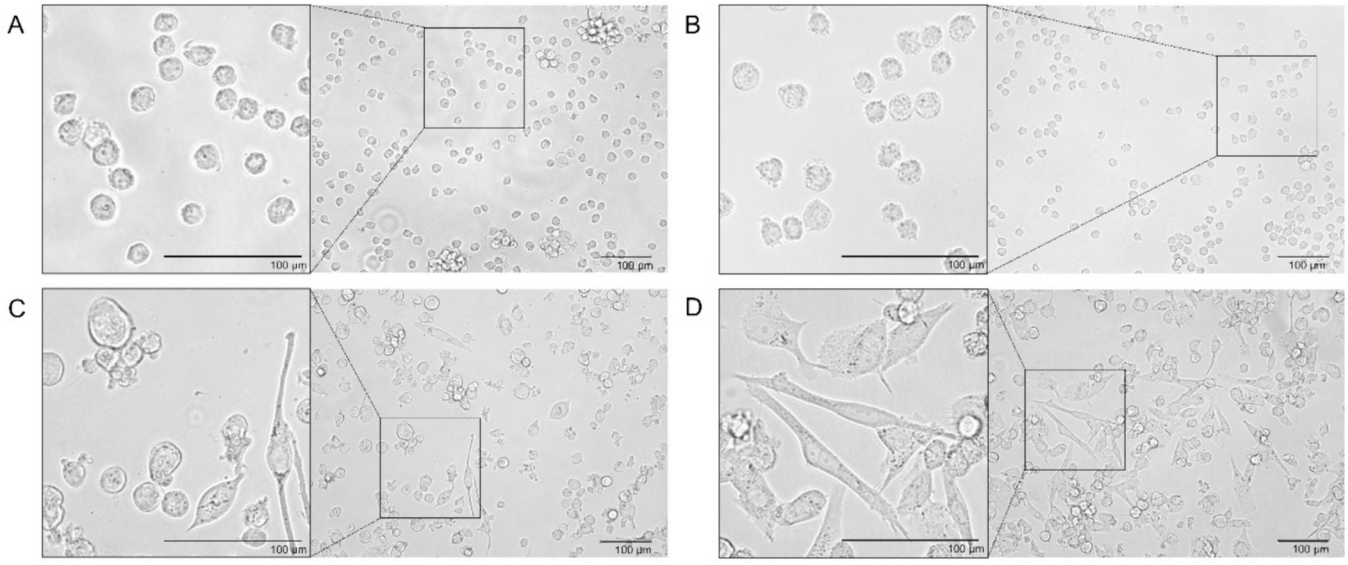

Fig. 1. PMA was required to induce the morphological characteristics of differentiated THP-1 macrophages. The micrographs show THP-1 cells exposed to the vehicle control (A), 10 nM 1,25D_3 (B), 5 nM PMA (C) and 5 nM PMA with 10 nM 1,25D_3 (D), following 96 h of differentiation are shown. Only PMA- and PMA with 1,25D_3-differentiated THP-1 cells showed distinct macrophage morphologies.

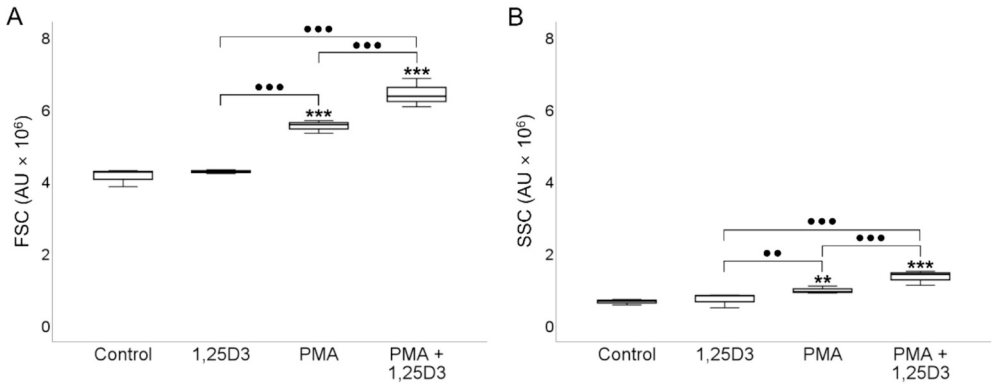

Fig. 2. THP-1 differentiation with 5 nM PMA in the presence of 10 nM $1,25D_3$ produced the greatest increase in cell size and granularity. The boxplots show forward scatter (FSC; A) and side scatter (SSC; B) measurements in arbitrary units (AU), following macrophage differentiation of THP-1 cells with 10 nM $1,25D_3$, 5 nM PMA, and 5 nM PMA with $1,25D_3$, over 96 h. Significant differences relative to the control (** $p < 0.010$, *** $p \leq 0.001$) and between differentiation conditions (●● $p < 0.010$, ●●● $p < 0.001$) were determined using a one-way ANOVA, followed by Fisher's LSD test. Error bars show the 95% CI ($n = 3$).

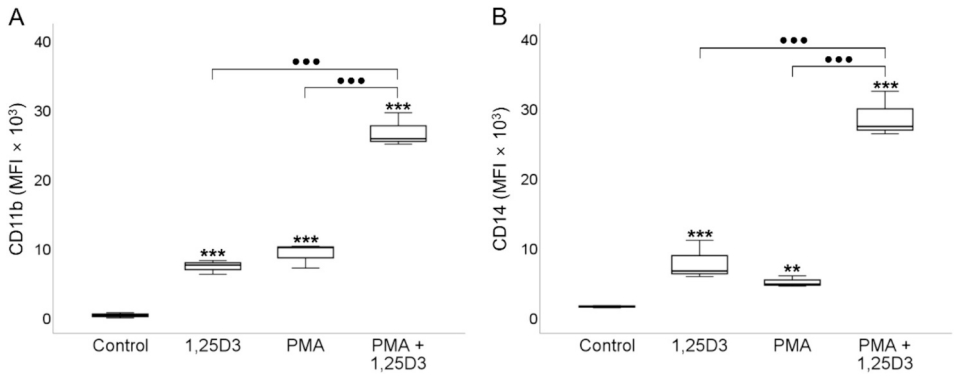

Fig. 3. THP-1 differentiation with 5 nM PMA in the presence of 10 nM $1,25D_3$ produced the greatest increase in CD11b and CD14 expression. The boxplots show CD11b (A) and CD14 (B) protein expression (MFI), following macrophage differentiation of THP-1 cells with 10 nM $1,25D_3$, 5 nM PMA, and 5 nM PMA with $1,25D_3$, over 96 h. Significant differences relative to the control (** $p < 0.010$, *** $p \leq 0.001$) and between differentiation conditions (●●● $p < 0.001$) were determined using a one-way ANOVA, followed by Fisher's LSD test. Error bars show the 95% CI ($n = 3$).

expression analysis of only the PMA-based differentiation protocols was performed. The success of differentiation was evaluated based on the differential expression of eighteen traditional, widely employed macrophage markers, whose expression is independent of macrophage polarization (Hesketh et al., 2017; Tedesco et al., 2018; Baxter et al., 2020; Mohd Yasin et al., 2022). These genes contribute to several aspects of macrophage biology including cell adhesion, phagocytosis, leukocyte extravasation, transcriptional regulation, and control of apoptosis. Monocyte-to-macrophage differentiation appeared to be successful in both the PMA- and PMA with $1,25D_3$–differentiated THP-1 cells based on the relative increase in expression observed for fourteen and fifteen of these markers in PMA- and PMA with $1,25D_3$-treated cells, respectively (Fig. 4, Table S3). Consistent with our previous results, treating THP-1 cells with PMA with $1,25D_3$ appeared to further increase the expression of the majority of these markers, most notably *CD14*, *C1QA*, *ITGAM*, *ICAM1*, *MAF*, and *MCL1*, in comparison to treating with PMA alone. The relative changes in the expression of twenty-two major tissue- and polarization-independent macrophage-specific markers, identified by Mulder et al. (2021) using single cell technologies in primary mononuclear phagocyte cells, were also evaluated (Fig. 4). The expression of sixteen and seventeen of these markers increased in the PMA- and PMA with $1,25D_3$–differentiated THP-1 cells, respectively. As seen with the traditional macrophage markers, the relative change in expression observed for most of these markers was generally greater in the PMA with $1,25D_3$-differentiated cells, especially for *CTSB*, *CD9*, *DAB2*, *LIPA*, and *SLCO2B1*.

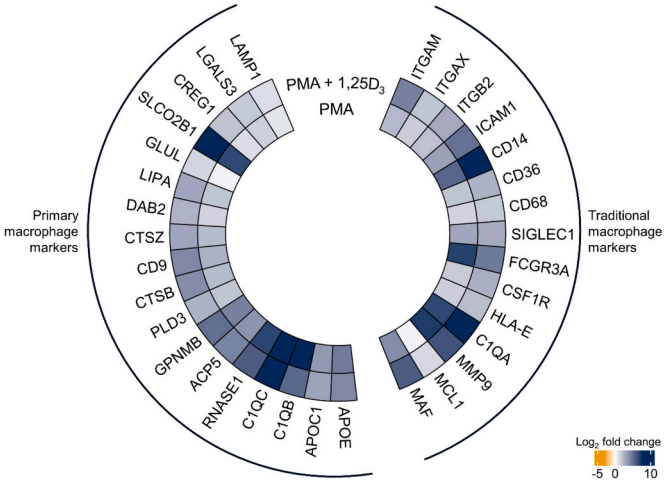

Fig. 4. THP-1 cells differentiated using 5 nM PMA with 10 nM $1,25D_3$ showed increased mRNA expression of traditional and primary macrophage markers relative to cells treated with PMA alone. Circular heatmap depicting the $\log_2$ fold change in expression of selected traditional and primary macrophage markers observed in each differentiation condition. In each case, the $\log_2$ fold change is given relative to the respective vehicle control.

3.4. THP-1 cells differentiated with either PMA or PMA with 1,25D$_3$ have the capacity for both M1- and M2 polarization

THP-1-derived macrophages may develop a propensity toward a particular polarization state because of the differentiation protocol employed (Park et al., 2007; Foey and Crean, 2013; Lund et al., 2016; Starr et al., 2018). As such, the differential expression of 56 and 43 genes associated with M1- and M2 polarization (Hesketh et al., 2017; Chistiakov et al., 2018; Tedesco et al., 2018; Orekhov et al., 2019; Baxter et al., 2020; Mohd Yasin et al., 2022; Rynikova et al., 2023), respectively, were evaluated (Table S4). Of these differentially expressed genes, there was a significant increase in the expression of nine M1-associated macrophage markers and a decrease in the expression of the cytokine encoding gene, *TNF* in PMA-differentiated THP-1 cells (Fig. 5). Similarly, a significant increase in the expression of twelve of these genes, and a decrease in the expression of the M1-associated MHC class II genes, *HLA-DPB1* and *HLA-DQB1,* was observed in PMA with 1,25D3- differentiated THP-1 cells. Notably, the expression of the potent pro-inflammatory cytokine, *CXCL10,* was the highest in the PMA-differentiated THP-1 cells. This was confirmed by RT-qPCR (Fig. S2). There was greater alteration in the expression of M2-polarization markers, with thirteen and nineteen genes showing a significant increase in expression in PMA- and PMA with 1,25D$_3$-differentiated THP-1 cells, respectively. In both the PMA- and PMA with 1,25D$_3$-differentiated THP-1 cells, there was a significant increase in the expression of frequently employed cell surface markers for macrophage polarization including M1-associated *TLR4* and *CD86*, and M2-associated *CLEC7A*, *MRC1* (CD206), *CD163*, *CD209*, *MSR1*(CD204), *PDCD1LG2*, and *CD274*. Overall, the PMA with 1,25D$_3$-differentiated THP-1 cells generally showed greater alteration in the expression of both M1- and M2 macrophage polarization-associated genes when compared to PMA alone.

3.5. Differentiation of THP-1 cells using PMA with 1,25D$_3$ enables polarization of the resulting macrophages into distinct M1- and M2-polarized states

In order to confirm the capacity for macrophage polarization in THP-1 cells differentiated using both PMA and PMA with 1,25D$_3$, macrophages derived using each of these differentiation protocols were subsequently exposed to LPS/IFN-γ for M1 polarization and IL-4/IL-13 for M2 polarization. The impact of treatment with these polarization protocols was assessed based on the protein expression of the M1- and M2 polarization markers, CD80 and CD206, respectively. Both the M1-polarized PMA- and PMA with 1,25D$_3$-differentiated THP-1 cells showed a significant increase in the expression of CD80 when compared to the control ($p < 0.010$) and M2-polarized macrophages (Fig. 6A-B; PMA: $p < 0.001$; PMA with 1,25D$_3$: $p < 0.010$). There was no significant difference in CD80 expression between control cells and either M2-polarized PMA- or PMA with 1,25D$_3$-differentiated THP-1 cells. Conversely, both the M2-polarized PMA- and PMA with 1,25D$_3$-differentiated THP-1 cells showed a significant increase in the expression of CD206 when compared to the control (Fig. 6C-D; PMA: $p < 0.010$; PMA with 1,25D$_3$: $p < 0.050$). However, THP-1 cells differentiated using PMA alone also exhibited significantly increased CD206 expression ($p < 0.050$) following M1 polarization, similar to that observed in M2-polarized cells. This was not observed in M1-polarized THP-1 cells differentiated using PMA with 1,25D$_3$, where CD206 expression in M1-polarized macrophages did not differ significantly from the control, but the expression of CD206 in M2-polarized macrophages was significantly greater than that in M1-polarized macrophages ($p < 0.050$).

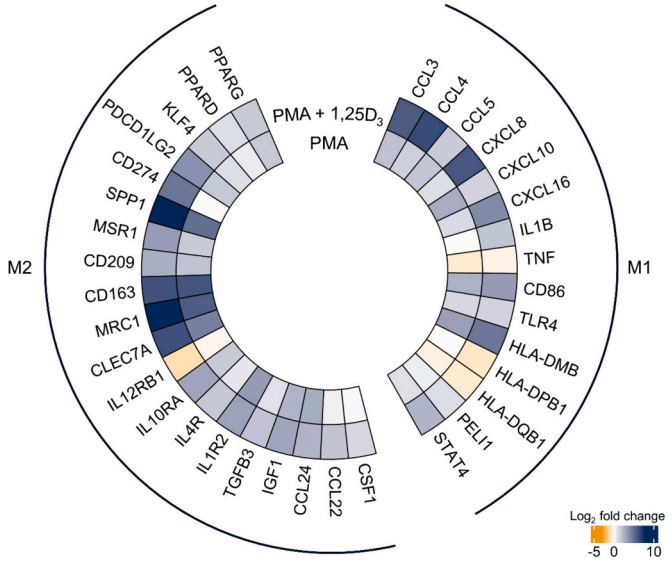

Fig. 5. Differentiation using PMA with 1,25D$_3$ resulted in increased expression of both M1- and M2 polarization markers compared to differentiation with PMA alone. Circular heatmap depicting the log$_2$ fold change in expression of M1- and M2 polarization markers in PMA- and PMA with 1,25D$_3$-differentiated THP-1 cells. In each case, the log$_2$ fold change is given relative to the relevant control.

4. Discussion

THP-1 cells are widely used in the study of monocyte-to-macrophage differentiation, macrophage activation, and macrophage function. Despite this, there is no standard protocol for the differentiation of THP-1 cells to THP-1-derived macrophages. Several groups have indicated that the differentiation protocol applied can produce vastly different THP-1-derived macrophages, with distinct gene expression profiles and phenotypic characteristics. These differences can result in a lack of comparability and reproducibility between studies (Park et al., 2007; Daigneault et al., 2010; Lund et al., 2016; Pinto et al., 2021). Here, we demonstrated that THP-1 cells treated with 10 nM 1,25D$_3$ does not produce the morphological and expression characteristics consistent with those observed in macrophages, despite significantly increasing the protein expression of macrophage surface markers CD11b and CD14. In contrast, low-dose PMA produced morphological and expression characteristics consistent with those observed in macrophages. These macrophage characteristics were more pronounced in cells differentiated using PMA in combination with 1,25D$_3$ than in cells differentiated with PMA alone. Additionally, THP-1 cells differentiated using PMA with 1,25D$_3$ showed increased mRNA expression of traditional and primary macrophage markers relative to cells treated with PMA alone. Moreover, differential gene expression analysis suggested that while either the PMA or PMA with 1,25D$_3$ differentiated THP-1 cells may loan themselves to either M1 or M2 macrophage polarization, only THP-1 cells differentiated using PMA with 1,25D$_3$ could be polarized into distinct M1- and M2 polarization states.

Monocyte-to-macrophage differentiation is morphologically characterized by increased cell size, increased cellular granularity, firm cellular adherence, and the development of cellular projections (Daigneault et al., 2010; Lund et al., 2016). Here, we demonstrate that PMA is required for the generation of these macrophage-associated morphological features - features that were more pronounced when PMA was combined with 1,25D$_3$. Despite being purported as an inducer of differentiation, 1,25D$_3$-differentiated THP-1 cells did not show the prototypical morphological characteristics of macrophages. Similarly, Rynikova et al. (2023) observed that exposure to 10 nM 1,25D$_3$ over seven days failed to produce macrophage morphology in THP-1 cells. Additionally, Daigneault et al. (2010) observed that a higher concentration of 100 nM 1,25D$_3$ also failed to produce macrophage morphology in THP-1 cells, suggesting that this effect may not be concentration dependent. Instead, it is possible that 1,25D$_3$ supports the differentiation state by altering the expression of genes associated with

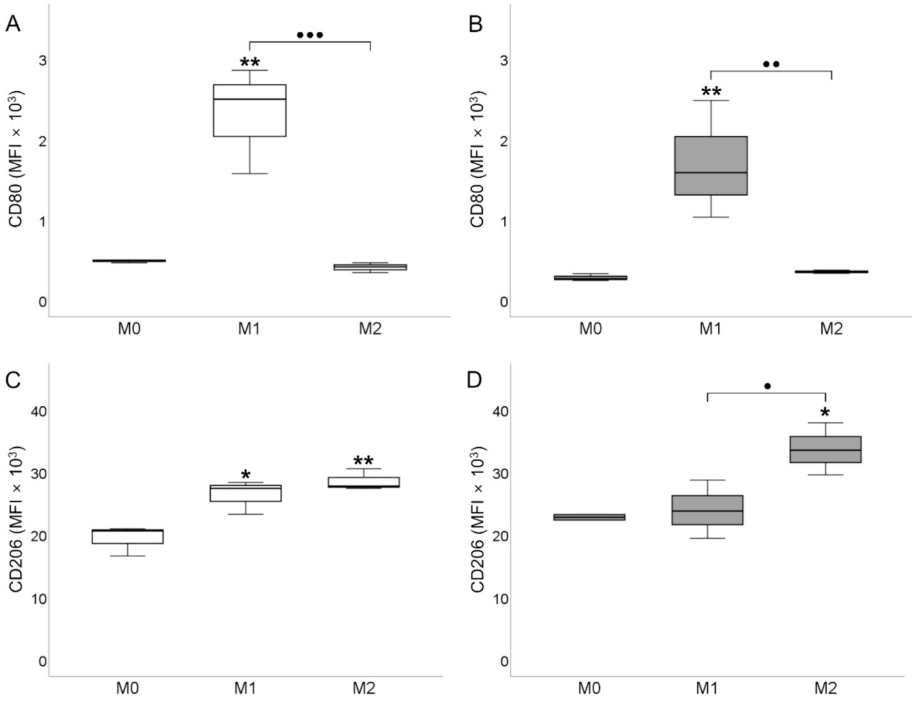

Fig. 6. Macrophages differentiated using 5 nM PMA with 10 nM $1,25D_3$ can be polarized into distinct M1- and M2 polarization states. The boxplots show changes in the expression of M1- (CD80) and M2 (CD206) surface markers in THP-1 macrophages differentiated with 5 nM PMA only (A and C; clear), or with 5 nM PMA in the presence of 10 nM $1,25D_3$ (B and D; filled), in the absence of additional stimuli (M0), or following treatment with either 20 ng/ml INF-γ and 100 ng/ml LPS (24 h, M1), or 20 ng/ml IL-13 and 50 ng/ml IL-4 (48 h, M2). Significant differences relative to M0 (* $p < 0.050$, ** $p < 0.010$) and between the M1 and M2-polarized states (• $p < 0.050$, •• $p < 0.010$, ••• $p < 0.001$) were determined using a one-way ANOVA, followed by Fisher's LSD test. Error bars show the 95% CI ($n = 3$).

differentiation. Indeed, here we show that while PMA is needed for THP-1 cells to adopt morphological characteristics associated with macrophages, these features are more pronounced in the PMA with $1,25D_3$-differentiated THP-1 cells. In agreement, Valdés López and Urcuqui-Inchima (2018) showed that PMA-based differentiation of U937 cells with $1,25D_3$ yielded macrophages with similar morphological characteristics as those observed in primary monocyte-derived macrophages – a feature that was not observed in the absence of $1,25D_3$. Thus, it is possible that $1,25D_3$ enhances the PMA-based differentiation of monocyte-like cell lines through its genomic effects.

For example, CD11b (encoded by *ITGAM*) and CD14 are commonly used cell surface macrophage markers, and it is thus expected that their expression will be increased with differentiation in THP-1 cells (Aldo et al., 2013; Spano et al., 2013; Hoffmann et al., 2019; Pinto et al., 2021). The protein expression of CD11b and CD14 was highest in the PMA with $1,25D_3$-differentiated THP-1 cells, implying that it was the most "mature" macrophage generated. Taken in isolation, the significant and comparable increase in CD11b and CD14 expression in both $1,25D_3$- and PMA-differentiated THP-1 cells implies that both protocols produce macrophages at a similar level of maturity. However, as the $1,25D_3$-differentiated THP-1 cells demonstrated no other phenotypic characteristics of differentiation, it is possible that these CD markers are not strictly indicators of differentiation, but rather reflect $1,25D_3$-mediated changes in expression outside of the differentiation process. Indeed, several studies have shown that the expression of *ITGAM* and *CD14* are directly impacted by the transcriptional activities of $1,25D_3$ when bound to its nuclear receptor, the vitamin D receptor, in monocytes (Moeenrezakhanlou et al., 2008; Neme et al., 2016; Nurminen et al., 2019). This could explain the increase in the protein expression of CD11b and CD14 in the absence of other macrophage characteristics in cells treated with $1,25D_3$ only. This would also account for why, in the absence of other techniques for the confirmation of differentiation, $1,25D_3$ is often cited as inducing differentiation.

Macrophages have several phenotypic and functional differences that separate them from monocytes. These include cell adhesion, phagocytosis, and pathogen interaction and removal. These differences are a consequence of the altered expression of specific protein-coding genes associated with these macrophage characteristics, which are thus frequently used as macrophage markers (Murray, 2017; Mohd Yasin et al., 2022). Many of these markers have direct roles in macrophage survival following differentiation, particularly the increased expression of *CSF1R*, *MAF*, and *MCL-1* (Busca et al., 2014; Hume et al., 2017; Mass et al., 2023). These genes were more highly expressed in the PMA with $1,25D_3$-differentiated THP-1 cells, suggesting that $1,25D_3$ may contribute to the increased longevity of these macrophages following differentiation.

Though the PMA- and PMA with $1,25D_3$-differentiated THP-1 cells showed similar increases in the expression of phagocytic receptors and controllers of lipid metabolism that mark differentiation, $1,25D_3$ combined with PMA resulted in greater mRNA expression of lysosome-associated genes marking differentiation. For example, there was an increased mRNA expression of the lysosomal membrane protein *LAMP1*, cathepsins, *CTSB* and *CTSZ*, and lysosomal homeostasis related gene, *PLD3,* in the PMA with $1,25D_3$-differentiated THP-1 cells compared to those differentiated with PMA alone. This indicates that $1,25D_3$ may support lysosome formation and function. Overall, these results indicated that while both differentiation protocols generated macrophages that may reflect their primary macrophage counterparts, $1,25D_3$ often augmented the mRNA expression of both the traditional and primary macrophage markers when compared to PMA alone. This suggests that the PMA with $1,25D_3$-differentiated THP-1 cells may represent more mature macrophages.

Given that THP-1 cells are most frequently differentiated using 100–200 nM PMA, (Daigneault et al., 2010; Chanput et al., 2014;), which is expected to produce macrophages skewed toward an M1-polarized state, it is interesting to note that the low-dose PMA used in

the current study did not yield any evidence of the skewed pro-inflammatory state previously reported (Park et al., 2007; Maeß et al., 2014; Lund et al., 2016; Riddy et al., 2018). Instead, both M1- and M2 polarization markers were observed in cells differentiated with low-dose PMA with or without $1,25D_3$. As with markers of differentiation, the altered expression of genes associated with polarization was more pronounced in PMA with $1,25D_3$-differentiated THP-1 cells. In this study, increases in the mRNA expression of M1-associated *CD86* and *TLR4,* and M2-associated *PDCD1LG2* (CD273), *CLEC7A*, *MRC1* (CD206), *CD163*, *CD209*, and *MSR1* were observed in both PMA- and PMA with $1,25D_3$-differentiated THP-1 cells (Tedesco et al., 2018; Yao et al., 2019; Fernandes et al., 2020; Mohd Yasin et al., 2022). However, the relative change in expression of M2-associated *PDCD1LG2*, *CLEC7A*, *MRC1*, and *MSR1* was notably higher in the PMA with $1,25D_3$-differentiated THP-1 cells. Similarly, expression of *CD274,* encoding a T-lymphocyte inhibitory ligand, was only significantly increased in PMA with $1,25D_3$-differentiated THP-1 cells. This may suggest that differentiation with a combination of PMA and $1,25D_3$ may result in an enhanced capacity for T-cell inhibition and phagocytosis.

The relative increase in the expression of several M1- and M2-associated cytokines and cytokine receptors with pro- and anti-inflammatory roles was also observed to be higher in PMA with $1,25D_3$-differentiated THP-1 cells (Palomino and Marti, 2015; Poeta et al., 2019; Korbecki et al., 2020; Ma et al., 2021; Lee et al., 2023). These included the M1-associated chemokines, *CCL3*, *CCL4*, *CXCL8*, and *CXCL16*, which are responsible for neutrophil-, natural killer cell-, monocyte-, and T-cell- chemotaxis (Ghafouri-Fard et al., 2021; Bao et al., 2023; Cambier et al., 2023). Conversely, $1,25D_3$ appeared to attenuate the increase in expression of *CXCL10* otherwise observed in PMA-differentiated cells. Given that *CXCL10* encodes a potent pro-inflammatory chemokine with anti-angiogenic properties, this is consistent with previous reports that suggest a role for $1,25D_3$ in balancing inflammatory responses (Korf et al., 2012; Scolletta et al., 2013; Zhao et al., 2017; Karin and Razon, 2018).

To investigate the potential influence of $1,25D_3$ on the subsequent polarization of the differentiated THP-1 cells, PMA- and PMA with $1,25D_3$-differentiated THP-1 cells were polarized to M1 macrophages using LPS/IFN-γ or M2 macrophages using IL-4/IL-13. Based on the expression of CD80 alone, M1 polarization of the PMA- and PMA with $1,25D_3$-differentiated THP-1 cells could be considered successful. However, M1-polarized, PMA-differentiated THP-1 cells also showed significantly increased CD206 expression compared to M0 macrophages that was not significantly different from that observed in M2-polarized macrophages. This was not the case for PMA with $1,25D_3$-differentiated THP-1 cells, which only showed significantly increased CD206 expression in M2-polarized macrophages. This suggests that $1,25D_3$ can aid in the commitment to a particular polarization state. Furthermore, the observation that either differentiation protocol can give rise to M2-polarized macrophages is useful, as this indicates that the low PMA concentration used in this study did not affect M2 polarization, as has previously been observed with higher concentrations of PMA (Maeß et al., 2014; Rynikova et al., 2023).

5. Conclusion

Taken together, our results suggest that $1,25D_3$ alone is not capable of producing a THP-1-derived macrophage, this was only achieved with PMA. As such, the use of common macrophage markers that are not primary $1,25D_3$ targets, including CD11c (encoded by *ITGAX*) or CD68, should be considered when differentiating THP-1 cells in the presence of $1,25D_3$. The use of low concentrations of PMA for the differentiation of THP-1 cells produced macrophages that did not exhibit the heightened inflammatory state induced by higher PMA concentrations. This PMA-induced THP-1 macrophage phenotype was augmented by the addition of $1,25D_3$. Thus, while both models explored herein would likely provide an efficacious means to study a range of macrophage immune functions, the use of the PMA with $1,25D_3$ differentiation protocol would provide a more physiologically relevant alternative, whilst retaining the benefits of a low PMA concentration. It has previously been illustrated that differentiation of THP-1 cells with lower PMA concentrations allows for more accurate study of intracellular and extracellular infection, with a particular focus on intracellular replication and control, responsiveness to bacterial stimuli, phagocytosis, and cytokine production (Park et al., 2007; Lund et al., 2016; Starr et al., 2018). Likewise, it has been indicated that the use of lower PMA concentrations in THP-1 cells produces a more accurate model for the investigation of NLRP3 inflammasome formation and cytokine production, known contributors to several auto-inflammatory disorders (Pinto et al., 2021; Giambelluca et al., 2022). Based on the enhanced expression of both M1- and M2-associated macrophage markers, the use of a lower PMA concentration, particularly when combined with $1,25D_3$, could further support the polarization of THP-1-derived macrophages into either M1- or M2-polarized macrophages, suggesting that the combined protocol will support studies aimed at investigating pro- and anti-inflammatory production in response to infection.

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CRediT authorship contribution statement

Bronwyn A. Mol: Writing – original draft, Visualization, Investigation, Data curation. **Janet J. Wasinda:** Writing – review & editing, Investigation, Data curation. **Yi F. Xu:** Writing – review & editing, Investigation, Data curation. **Nikki L. Gentle:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Vanessa Meyer:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2024.113716.

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