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Master's Thesis

Evaluation of calcitriol modulation on phagocytic activity and cytokines expression in U937-derived macrophages

Supervisor:

Prof. Pier Paolo Sainaghi

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Candidate:

Daniela Verrascina

Matricula 20035889

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1. SUMMARY

Rationale of the study: Vitamin D is a secosteroid hormone traditionally associated with calcium and phosphate homeostasis. However, increasing evidence has highlighted its involvement in the regulation of immune responses, particularly through the modulation of macrophage function and plasticity. Macrophages play a central role in innate immunity and can acquire distinct phenotypes in response to environmental stimuli. The aim of this study was to investigate the effects of calcitriol on U937-derived macrophages by evaluating cell viability, phagocytic activity and the expression of inflammatory cytokines.

Methods: U937 cells were differentiated into macrophages and treated with different concentrations of calcitriol (0.05 nM, 0.1 nM, 1 nM, 5 nM and 10 nM). Cell viability was assessed by Crystal Violet assay, while phagocytic activity was evaluated using a fluorescence-based phagocytosis assay. Based on the phagocytosis results, 0.1 nM calcitriol was selected for gene expression analyses. Macrophages were polarized into M0, M1-like and M2-like phenotypes using specific stimuli and the expression of TNF-α, IL-6 and IL-10 was evaluated by RT-qPCR.

Results: Calcitriol treatment significantly increased cell viability following exposure to 10 nM calcitriol and enhanced phagocytic activity at 0.1 nM and 1 nM concentrations. Morphological observations confirmed the presence of both M1-like and M2-like macrophage phenotypes after differentiation. Gene expression analysis revealed that treatment with 0.1 nM calcitriol significantly reduces TNF-α expression in M1-like and M2-like macrophages, whereas its expression result to increase in M0 macrophages. No significant differences were observed in IL-6 expression among the experimental groups. In contrast, IL-10 expression was significantly increased in M0 and M2-like macrophages, with the highest expression levels detected in M2-like cells.

Conclusions: The results suggest that calcitriol modulates several functional and molecular aspects of macrophage biology, including cell viability, phagocytic activity and cytokine expression. Although some findings differed from those previously reported in the literature, the overall data indicate a potential role of calcitriol in promoting anti-inflammatory macrophage responses. Further studies are required to clarify the mechanisms underlying these effects and to better define the contribution of vitamin D to macrophage polarization and immune regulation.

2. INTRODUCTION

2.1 Immune System

The immune system is a complex network composed of organs and tissues along with specific cells and soluble mediators that work together to generate a protective response against pathogens (e.g. viruses, bacteria and parasites) and abnormal cells (e.g. tumours and transplanted cells). In order to ensure optimal immune function, it is necessary a harmonious interplay between the innate and adaptive immune systems [1]. The innate immune response react rapidly and non specifically upon encountering a pathogen [2], while the adaptive immune response is slower because it is characterised by the presence of specialised immune cells, including B and T cells. These cells adapt and respond to foreign antigens to the body [3], by also building up immunological memory essential to exert a rapid response upon reencountering the antigen [2].

However, the first line of defence is represented by the innate immune system, which is distributed across various tissues and organs throughout the body and it comprises a variety of immune cells, including granulocytes, dendritic cells, monocytes, and macrophages [1]. Innate immune cells recognise pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs). These receptors can also be activated by endogenous damage-associated molecular patterns (DAMPs), which are released by injured cells during sterile inflammation. Activation of PRRs in innate immune cells rapidly induces the secretion of pro-inflammatory cytokines, such as interleukin (IL)-1β and IL-6, and tumour necrosis factor (TNF). These inflammatory mediators stimulate the presentation of antigens to the adaptive immune system, thereby activating a specific immune response [4].

Among the different populations of innate immune cells, macrophages are particularly important because they contribute both to pathogen clearance and to the regulation of inflammatory processes [3]. By releasing cytokines, chemokines and other signalling molecules, macrophages actively participate in different stages of the immune response, from the initial recognition of danger signals to the resolution of inflammation and tissue repair. As key components of innate immunity, macrophages are involved in maintaining tissue homeostasis and coordinating immune responses under both physiological and pathological conditions. Therefore, a deeper understanding of macrophage biology is essential for investigating the mechanisms that control inflammation and immune homeostasis [5].

2.2 Macrophages

Macrophages are generally divided into tissue-resident and monocyte-derived macrophages (MDMs), depending on their origin and location. Tissue-resident macrophages, such as Kupffer cells in the liver or microglia in the brain, arise during embryonic development and settle in tissues before birth. Therefore, they perform specialised homeostatic and surveillance functions. MDMs, on the other hand, derive from circulating monocytes generated in the bone marrow from haematopoietic stem and progenitor cells [5]. Monocytes themselves are a heterogeneous population, comprising classical, intermediate, and non classical subsets, each characterized by distinct migratory patterns, cytokine profiles, and immune functions. Different types of monocytes reflect their diverse roles in inflammation and tissue homeostasis. In response to tissue damage, infection or inflammation, monocytes are recruited to the affected site, where they differentiate into macrophages or dendritic cells [6].

Once differentiated, macrophages acquire highly specialized functions that enable them to respond to the requirements of different tissues and microenvironments. Macrophages are essential immune cells due to their elevated plasticity. These cells are widely distributed across tissues and organs, where they adapt to local environmental signals, acquiring distinct phenotypic and functional profiles in the process [5].

Macrophages contribute to both tissue maintenance and host defence by carrying out several essential activities, including phagocytosis, efferocytosis and antigen presentation. Phagocytosis enables the uptake and degradation of pathogens and particulate matter [7], while efferocytosis ensures the efficient clearance of apoptotic cells, thereby promoting the resolution of inflammation [8]. Additionally, macrophages process internalized material and present antigenic peptides via major histocompatibility complex class II (MHC-II) molecules, thereby linking innate immune responses to the activation of adaptive immunity [9].

One of the most distinctive characteristics of macrophages is their ability to modify their functional behaviour in response to environmental cues. Depending on the surrounding signals, they can acquire different activation states that are generally categorized as pro-inflammatory M1 or anti-inflammatory M2 phenotypes (**Figure 1**). M1-type macrophages are typically associated with inflammatory environments characterized by toll-like receptor (TLR) activation and interferon (IFN) signalling. They promote T helper (Th)1 immune responses and act as a first line of defence against microorganisms by enhancing pro-inflammatory pathways, eliminating intracellular pathogens, and

producing reactive oxygen species (ROS), which can contribute to tissue damage and impair tissue repair processes [10].

M1 macrophages are typically activated by bacterial lipopolysaccharide (LPS) and IFN-γ, triggering the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. In addition, they generate high levels of ROS and reactive nitrogen species (RNS), upregulate surface markers such as cluster differentiation (CD)40, CD80, CD86, and MHC-II, and perform key functions including pathogen elimination, phagocytosis, and clearance of senescent or damaged cells [11]. The expression of MHC-II and co-stimulatory molecules plays a crucial role in initiating adaptive immunity. In particular, CD80 and CD86 are cell surface glycoproteins found on various antigen-presenting cells that interact with CD28 on naïve T cells, thereby enhancing their proliferation and effector activity. Similarly, the CD40 receptor, engages with its ligand CD40L on activated $CD4^+$ T cells to stimulate Th1 responses [10].

Conversely, M2-type macrophages, also known as reparative macrophages, are involved in promoting Th2 responses. Their anti-inflammatory properties, along with the strong phagocytic capacity, enable the removal of apoptotic cells and cellular debris, support tissue repair, wound healing and angiogenesis [12]. The development of anti-inflammatory M2 macrophages is induced by IL-4 and IL-13 and is associated with the expression of anti-inflammatory mediators, including IL-10 and transforming growth factor (TGF)-β, as well as surface markers such as CD163, CD204 and CD206 [13]. In particular, CD163 is involved in haemoglobin clearance and anti-inflammatory responses, while CD206 mediates the recognition and uptake of pathogen-associated molecules, supporting immune regulation and tissue homeostasis [14].

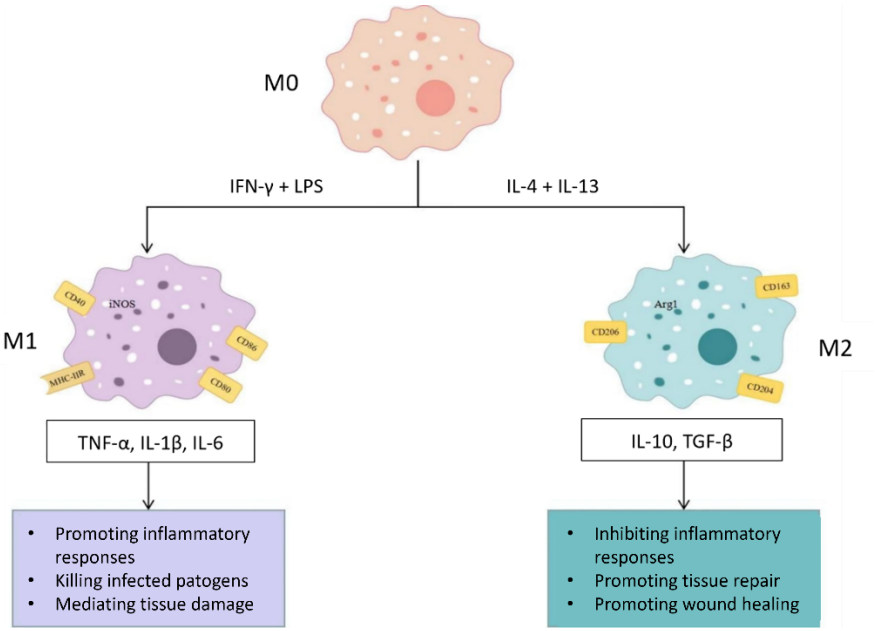

Figure 1: M1-type and M2-type macrophage polarization. M0 macrophages can differentiate into pro-inflammatory M1 macrophages following stimulation with IFN-γ and LPS or into anti-inflammatory M2 macrophages in response to IL-4 and IL-13. The figure also summarizes the main markers, cytokines and biological functions associated with each phenotype. Modified with Biorender.com [10].

M2 macrophages encompass several subtypes, including M2a, M2b, M2c, and M2d, which arise in response to distinct stimuli and display diverse functional profiles. Among these, M2a macrophages are primarily involved in the clearance of cellular debris and the promotion of tissue repair through the release of pro-fibrotic factors. In contrast, M2b and M2c macrophages are often described as regulatory subsets that contribute to the maintenance of immune balance and the restoration of tissue homeostasis, while the M2d subtype has been associated with processes related to wound healing and tissue remodelling [15].

During the progression of an immune response, macrophage polarization remains a highly dynamic process. M1 macrophages dominate the early stages to promote pathogen clearance and inflammatory signalling, whereas a subsequent shift toward M2 phenotypes facilitates the resolution of inflammation and tissue repair [16]. The plasticity of macrophages allows them to adapt their functional profile in response to environmental stimuli, and imbalances in M1/M2 ratios have been associated with the development and progression of chronic inflammatory and autoimmune diseases. In this context, excessive M1 activity can perpetuate tissue damage, whereas dominant M2 responses may lead to fibrosis and impaired immune defence [11]. Metabolic reprogramming is a key mechanism underlying macrophage polarization, with distinct metabolic pathways supporting either pro-inflammatory or reparative phenotypes. In particular, M1 macrophages predominantly

rely on glycolysis, whereas M2 macrophages preferentially utilize oxidative phosphorylation and fatty acid oxidation, supporting their distinct pro-inflammatory and reparative functions [17]. This metabolic flexibility is closely linked to macrophage function in tissue homeostasis, immunity, and response to injury [18].

Several *in vitro* cell models have been frequently used to investigate the molecular mechanisms underlying macrophage differentiation and polarization. Specifically, human monocytic cell lines are pivotal experimental tools to understand macrophage biology and develop novel therapeutic strategies [19]. These experimental models allow for the study of monocyte differentiation into macrophages in response to specific environmental stimuli [20], while also investigating their functional plasticity [21].

Among these, the human monocytic leukemia cell line Tohoku Hospital Pediatrics-1 (THP-1) and the human histiocytic lymphoma cell line U937 are widely used as *in vitro* models in macrophage research [19]. These cells can be stimulated to differentiate into macrophage-like cells and are commonly employed to analyse immune responses, polarization, and signalling pathways *in vitro*[22].

Monocytic cell lines represent a valuable tool for macrophage studies, as they provide a consistent source of precursor cells and allow experiments to be performed under reproducible conditions. In addition, their standardized features contribute to greater comparability between independent studies. However, choosing an appropriate *in vitro* model requires a balance between practical use and functional accuracy. While immortalized cell lines are easy to handle and provide consistent results, they may differ from *in vivo* behaviour, especially in complex or long-term immunological studies [19]. Despite their limitations, monocytic cell lines remain valuable tools for studying the molecular response to several immunomodulatory molecules, such as vitamin D, which may regulate macrophage activation, differentiation and functional responses [18].

2.4 Vitamin D

Vitamin D is a secosteroid hormone primarily involved in the regulation of calcium and phosphate metabolism, thereby contributing to bone mineralization and the maintenance of skeletal health. Beyond its classical role in bone homeostasis, vitamin D is involved in a variety of physiological processes that contribute to overall health [23].

Vitamin D can be obtained either through dietary intake or through endogenous synthesis. Regarding dietary sources, vitamin D is available in two main forms: vitamin D_2 or ergocalciferol found in plants, fungi and yeast, and vitamin D_3 or cholecalciferol primarily derived from animal sources [24].

In humans, vitamin D is produced in the skin starting from 7-dehydrocholesterol that, upon ultraviolet B (UVB) exposure, is converted to previtamin D_3 (**Figure 2**). This molecule is then transformed into cholecalciferol through thermal isomerisation [25]. In the bloodstream, cholecalciferol is transported through the vitamin D binding protein (VDBP), and, to a lesser extent, albumin, to the liver where it is hydroxylated by the enzyme cytochrome P450 (CYP)2R1, alternatively termed 25-hydroxylase, to the most abundant circulating form of vitamin D, 25-hydroxyvitamin D also known as calcidiol [26].

Calcidiol is commonly used to assess vitamin D status because it is the predominant form found in the bloodstream. Serum concentrations below 20 ng/mL generally indicate deficiency, whereas levels between 30 and 60 ng/mL are considered adequate for most individuals [27].

This metabolite is subsequently transported by VDBP to the kidneys, where it undergoes a second hydroxylation step mediated by the enzyme CYP27B1 (1α-hydroxylase), leading to the formation of the active form, 1,25-dihydroxyvitamin D or calcitriol [24]. In the kidneys, this hydroxylation process is strictly controlled by parathyroid hormone (PTH), as well as serum calcium and phosphate levels. This regulatory network ensures calcium homeostasis, primarily by modulating intestinal calcium absorption. In cases of insufficient calcitriol levels, the body prioritizes systemic calcium balance by promoting bone resorption, whereby calcium is extracted from the bone matrix, to compensate for reduced intestinal absorption. Once formed, calcitriol circulates throughout the body and exerts a variety of biological effects[28].

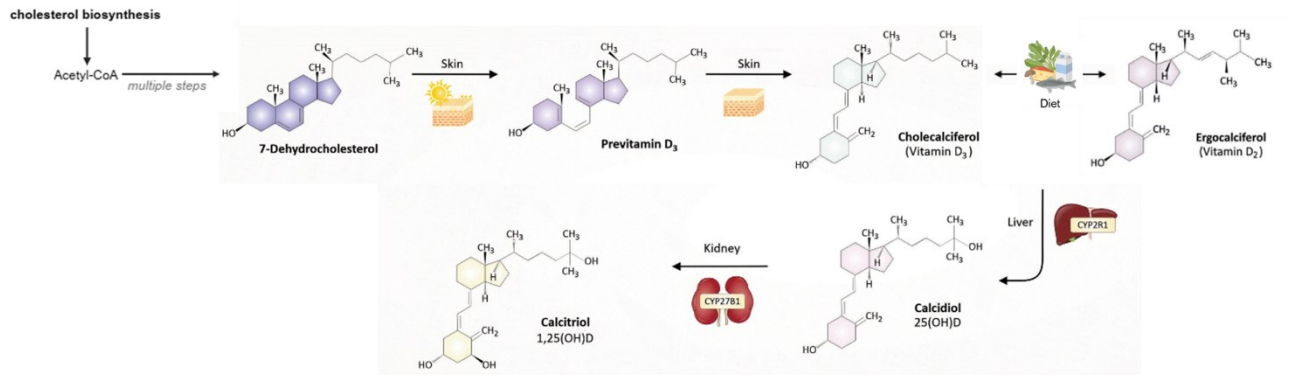

Figure 2: Schematic representation of vitamin D synthesis and activation. Following exposure to UVB radiation, 7-dehydrocholesterol present in the skin is converted into previtamin D_3 and subsequently into cholecalciferol (vitamin D_3). Vitamin D may also be obtained through the diet as vitamin D_3 or ergocalciferol (vitamin D_2). Both forms are first converted in the liver into 25-hydroxyvitamin D (calcidiol) and are then further processed in the kidney by CYP27B1 to generate 1,25-dihydroxyvitamin D (calcitriol), the hormonally active form of vitamin D. Modified using BioRender.com[24].

The biological effects of calcitriol are mediated by the vitamin D receptor (VDR), a member of the nuclear receptor superfamily expressed in many different tissues, including immune cells. Upon ligand binding, the VDR forms a heterodimer with the retinoid X receptor (RXR) and translocate into the nucleus. This complex then binds to specific DNA sequences known as vitamin D response elements (VDREs), regulating the expression of genes involved in processes such as cell proliferation, differentiation, and metabolism. In addition, vitamin D can also induce rapid non-genomic effects through membrane-associated VDRs, further contributing to the regulation of cellular functions [29].

Vitamin D deficiency is very common worldwide and has been associated not only with bone diseases such as rickets and osteoporosis but also with an increased risk of metabolic and autoimmune disorders [30]. Due to the high prevalence of vitamin D deficiency, especially in individuals with limited sun exposure or certain health conditions, maintaining adequate levels is essential. In this context, exogenous supplementation of vitamin D can be useful since this molecule is involved in several physiological processes including immune regulation, metabolic balance and cardiovascular function [27,31].

2.5 Immunomodulatory effects of Vitamin D

In addition to its systemic functions, vitamin D influences several cellular processes, including proliferation, differentiation and inflammatory responses [32].

The effect of vitamin D is antiproliferative, in fact it induces the arrest of the cell cycle in the G1/S checkpoint through the up-regulation of cyclin-dependent kinase inhibitors, such as p21 and p27 [33].

However, in specific microenvironments, such as mesenchymal stem cell niches or during tissue repair processes, vitamin D can induce proliferation by stimulating cellular self-renewal and preserving genomic stability via telomere maintenance [34].

Besides regulating cell proliferation, vitamin D also contributes to cellular differentiation by promoting the transition from immature cells to more specialized phenotypes. This mechanism is mainly present in the immune system, where calcitriol not only inhibits cell growth, but orchestrates the differentiation of myeloid and lymphoid precursors into cellular subtypes [35]. In fact, vitamin D exerts significant effects on the innate immune system by enhancing the recruitment and maturation of myeloid progenitors into fully functional monocytes and macrophages, while simultaneously reprogramming them toward an anti-inflammatory M2 phenotype [18].

Specifically, these cells of the innate immune system possess the enzymatic machinery necessary for the local bioactivation of vitamin D. Unlike the systemic renal process, macrophages and monocytes express the enzyme CYP27B1 (1α-hydroxylase) in response to inflammatory signals, such as TLR activation or IFN-γ exposure. This expression allows them to convert circulating calcidiol into its active form, calcitriol, directly at the site of interest. Through this local activation mechanism, monocytes and macrophages can directly enhance their antimicrobial activity at sites of infection [35]. Upon binding to the VDR-RXR complex, calcitriol induces the transcription of endogenous antimicrobial peptides, specifically cathelicidin (LL-37) and β-defensin 2. These molecules exert their protective effects by directly disrupting the cell membranes of bacteria and viruses, or by initiating signalling cascades that neutralize infected cells. In addition, vitamin D also regulates autophagy, a conserved degradation process essential for clearing intracellular infections. Through the transcriptional induction of autophagy-related proteins, such as Autophagy Related 5 (Atg-5) and Beclin-1, calcitriol promotes the formation of autophagosomes that entrap and eliminate viral particles and resistant bacteria. Furthermore, while enhancing these microbicidal activities, vitamin D modulates the inflammatory response by inhibiting the overproduction of pro-inflammatory cytokines, such as TNF-α and IL-6, thereby protecting from excessive tissue damage[35].

Beyond these immediate effects, the local production of calcitriol can induce epigenetic changes in monocytes and macrophages. This alteration not only affects their differentiation into specialized subtypes but also affects immunological memory, thereby improving the host's capacity to react to future infectious threats [36].

Despite its primary impact being on the innate compartment, calcitriol also serves as a vital bridge to adaptive immunity by influencing the activity of dendritic cells (DCs). Under the influence of vitamin D, DCs are maintained in a tolerogenic or "immature" state, characterized by a reduced expression of MHC-II and co-stimulatory molecules such as CD80 and CD86 [37]. This modulation effectively limits the capacity of DCs to trigger an overactive adaptive response. Consequently, these changes in the dendritic microenvironment significantly impact T-cell polarization.

Vitamin D reduces the differentiation and proliferation of Th cells, specifically targeting the pro-inflammatory Th1 and Th17 subpopulations, while favouring a shift toward Th2 differentiation and IL-4 production. One of the hallmark mechanisms through which vitamin D maintains immune homeostasis is the promotion of regulatory T cells (Tregs). These specialized cells act as potent suppressors of inflammation, playing a pivotal role in inhibiting the activation of Th1 and Th17 cells and maintaining self-tolerance [38].

Furthermore, vitamin D exerts a direct inhibitory effect on B-cell proliferation and their differentiation into plasma cells, thereby modulating antibody production. By orchestrating these complex interactions between dendritic cells and lymphocytes, vitamin D acts as a systemic checkpoint that prevents the transition from an acute innate response into chronic, immune-mediated tissue damage [39].

Based on the current knowledge, vitamin D plays a key role in the immune system by modulating macrophage function and plasticity. Its local activation enhances antimicrobial responses while promoting a shift toward an anti-inflammatory profile. This dual effect allows vitamin D to support host defence while limiting excessive immune activation, thereby contributing to immune homeostasis [35].

3. AIM OF THE PROJECT

The aim of this project was to investigate the effects of calcitriol on macrophage biology using a U937-derived macrophage model. Specifically, the study focused on evaluating the impact of calcitriol treatment on cell viability, phagocytic activity and the expression of inflammatory cytokines in M0, M1-like and M2-like macrophages. Through these analyses, the project aimed to further characterize the immunomodulatory properties of vitamin D and its potential role in regulating macrophage function.

4. MATERIAL AND METHODS

4.1 Cell lines and cell culture

The human promonocytic cell line U937 was used for all experiments. U937 cells, originally established from a human histiocytic lymphoma, represent an *in vitro* model of immature monocytic cells and are widely employed in studies investigating monocyte/macrophage biology and differentiation. Undifferentiated U937 cells were maintained in suspension culture throughout the experiments (**Figure 3**). Cells were cultured in RPMI 1640 GlutaMAX™ (Cat. #72400-021, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) medium supplemented with 10% heat-inactivated foetal bovine serum (FBS, Cat. #A5256701, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA). Cultures were maintained in a humidified incubator at 37°C with 5% CO_2, and the medium was renewed every 2 -3 days to maintain exponential growth.

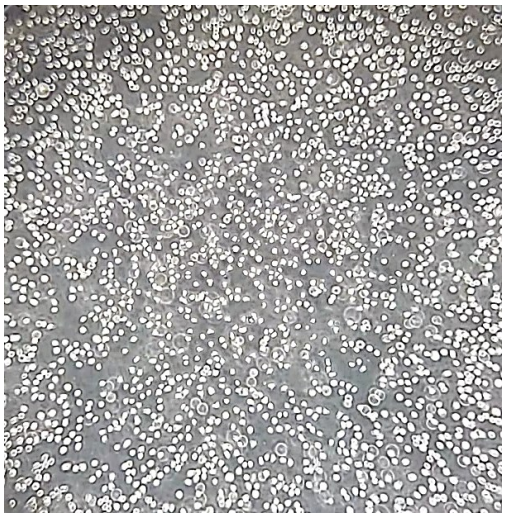

Figure 3: The figure shows a culture of U937 cell line.

4.2 Cell treatments

U937 cells were subjected to various treatment conditions depending on the experimental design. To induce differentiation and cell adherence, cells were pre-treated with 20 ng/ml of phorbol 12-myristate 13-acetate (PMA) before any subsequent experiment.

Cells were exposed to various concentrations (0.05 nM, 0.1 nM, 1 nM, 5 nM and 10 nM) of calcitriol (Cat. #HY-1002, MedChemExpress, Monmouth Junction, NJ, USA). The compound was initially dissolved in dimethyl sulfoxide (DMSO) to obtain a 1 mM primary stock solution. Experimental working solutions were then prepared via serial dilutions in culture medium. Intermediate stocks of

1 µM and 100 nM were generated; the lower concentrations (0.05 nM, 0.1 nM and 1 nM) were obtained from a 10 nM dilution, while the higher concentrations (5 nM and 10 nM) were prepared from the 100 nM stock.

4.3 Crystal Violet Assay

To quantify the total adherent cell viability after treatment, a Crystal Violet staining assay was performed. U937 cells were seeded in 12-well plates at a density of 500,000 cells/well in a final volume of 1 mL. Cells were treated in duplicate with untreated control and different concentrations of calcitriol (0.05 nM, 0.1 nM, 1 nM, 5 nM and 10 nM).

After 24 hours of incubation, the culture medium was removed, and each well was washed with 1x phosphate-buffered saline (PBS). The adherent cells were then fixed by adding ice-cold methanol and incubating the plates at -20°C for 30 minutes. Following fixation, the methanol was removed, and cells were stained with 400 µL of 0.5% Crystal Violet solution (Cat. #491502, Carlo Erba Reagents, Cornaredo, MI, Italy) in 20% methanol. The plates were incubated for 30 minutes at room temperature in the dark.

After the staining period, the excess dye was removed by washing the wells with deionized water (H_2O). The plates were then photographed using Leica DMi1 inverted microscope (Leica Microsystems, Wetzlar, Germany) and allowed to air-dry completely. To quantify the stained biomass, 400 µL of 10% acetic acid was added to each well and incubated for 20 minutes to solubilize the dye. The absorbance was subsequently measured at 570 nm using the 2030 Multilabel Reader VICTOR X4 (Cat. #2030-0040, PerkinElmer, Waltham, MA, USA). The final results were expressed as a percentage of relative cell viability (%) relative to untreated control. The mathematical normalization was performed according to the following equation:

$$\text{Cell viability \%} = \left(\frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

4.4 Phagocytosis Assay via Incucyte

To evaluate the phagocytic activity of differentiated U937 cells, an Incucyte® live-cell imaging assay was performed using pHrodo™ *E. Coli* BioParticles™ conjugate (Cat. #P35361, Invitrogen™ by Thermo Fisher Scientific).

Cells were seeded in 96-well plates at a density of 50,000 cells/well in a final volume of 200 µL. The initial treatment phase followed the same protocol described for the viability assay: after differentiation, cells were treated with calcitriol (0.05 nM, 0.1 nM, 1 nM, 5 nM and 10 nM) for 24 hours, washed with PBS 1x, and replenished with fresh medium containing the respective vitamin D concentrations for an additional 72 hours.

Following this 72-hour period, the culture medium was completely removed. To each well, 90 µL of fresh medium containing the respective calcitriol concentration and 10 µL of pHrodo™ *E .Coli* BioParticles™ suspension was added to reach a final volume of 100 µL per well. The plates were then returned to the Incucyte® SX5 system for 24 hours.

Phagocytic activity was quantified and analysed using the Incucyte® SX5 software (Fluorescent Dye mode). The system captured red fluorescence signals, which are activated specifically upon internalization of the BioParticles within the acidic environment of phagosomes. The phagocytic index was calculated based on the total red fluorescence object area (μm^2/Image) masked per well and all samples were analysed in triplicate.

4.5 Cell seeding and treatments for Real-Time qPCR

U937 cells were seeded in 12-well plates at a density of 500,000 cells/well in a final volume of 1 mL. Cells were differentiated for 24 hours with 20 ng/ml PMA in presence or absence of 0.1 nM calcitriol.

After 24 hours of incubation, the culture medium was removed, and wells were washed with 1x PBS. Cells were then replenished with 1 mL of fresh complete medium containing their respective treatments: standard medium (control) or 0.1 nM calcitriol.

Following additional 72 hours of incubation, the medium was removed, cells were washed with 1x PBS and incubated in 1 mL of low-serum medium (RPMI-1640 supplemented with 1% FBS) containing the respective treatment (standard medium or 0.1 nM calcitriol) for a 24-hour starvation period.

Subsequently, macrophage polarization was induced for 48 hours in a final volume of 1 mL of complete RPMI-1640 medium (10% FBS), maintaining the respective calcitriol treatment. The polarization conditions were defined as follows:

- M0 Phenotype: Cells received only fresh complete RPMI-1640 medium.
- M1 Phenotype (Pro-inflammatory activation): Cells were stimulated with LPS (100 ng/mL; Sigma-Aldrich), diluted from a 1 mg/mL stock solution resuspended in RPMI, and IFNγ (20 ng/mL; ImmunoTools), diluted from a 100 µg/mL stock solution.
- M2 Phenotype (Alternative activation): Cells were stimulated with IL-4 (20 ng/mL; ImmunoTools), diluted from a 10 µg/mL stock solution.

After the 48-hour polarization phase, cells were harvested to proceed with total RNA extraction. The schematic representation of the experimental procedure is presented in **Figure 4**.

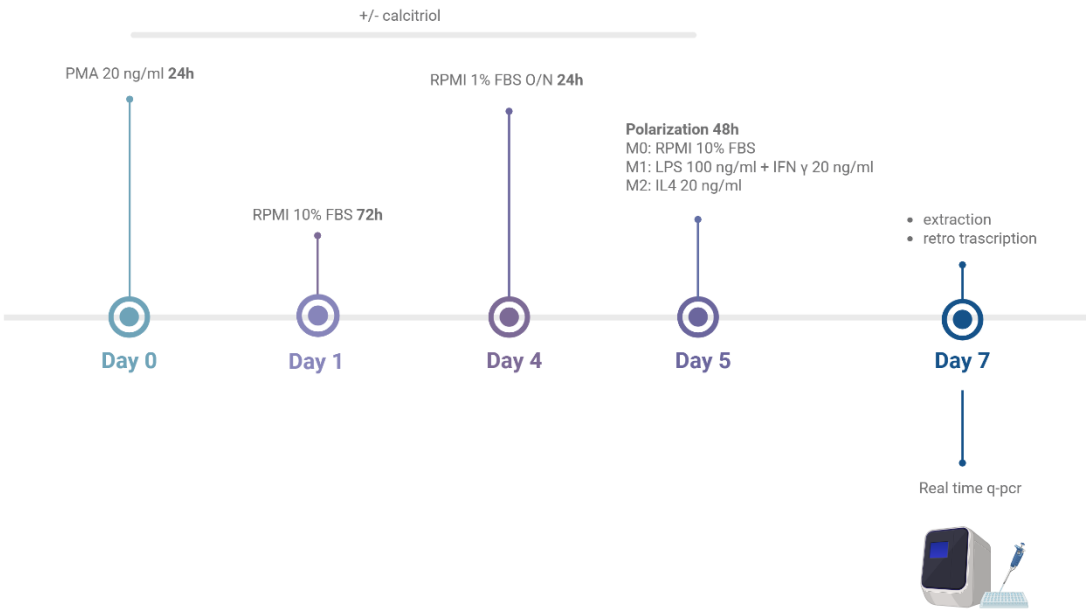

Figure 4: Schematic representation of the 7-day experimental workflow for RT-qPCR analysis. On Day 0 U937 cells were seeded and differentiated with PMA in the presence or absence of calcitriol (0.1 nM). After a 72h incubation in complete medium (Day 1) and a subsequent 24 h starvation period in 1% FBS medium (Day 4), macrophage polarization was induced for 48 h to obtain M0, M1 (LPS + IFN γ), and M2 (IL-4) phenotypes while maintaining calcitriol treatments (Day 5). On day 7 cells were harvested for total RNA extraction and subsequent reverse transcription. Figure created using BioRender.com.

4.7 RNA extraction

Total RNA was isolated from polarized U937 cells using NucleoZOL reagent (Macherey-Nagel GmbH & Co. KG, Düren, Germany), according to the manufacturer's instructions.

The extracted RNA was quantified using a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Cat. #ND-ONE-W, Thermo Fisher Scientific, Waltham, MA, USA). For quantification, 1 µl of RNase-free water was used as a blank, followed by the measurement of 1 µl of each eluted RNA sample. Purified RNA was immediately kept on ice for further processing or stored at -80 ° C for long-term preservation.

4.8 Reverse transcription

The extracted total RNA was reverse-transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Cat. #4368814, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

For each reaction, 500 ng of total RNA was combined with the reverse transcription master mix in a final volume of 20 µl. Reverse transcription was performed in a thermal cycler under the conditions shown in **Figure 5**. Synthesized cDNA samples were then stored at -30 °C until further use.

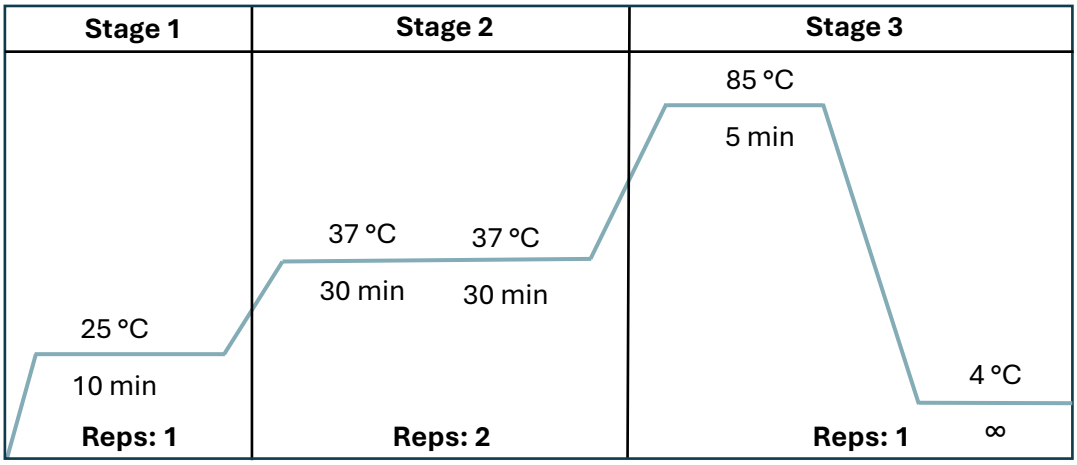

Figure 5: Thermal profile used for reverse transcription of total RNA into cDNA. The reaction included incubation at 25°C for 10 min, 37°C for 30 min, enzyme inactivation at 85°C for 5 min, and storage at 4°C.

4.9 Real-Time qPCR

Quantitative Real-Time PCR (RT-qPCR) was performed to determine the mRNA expression levels of the following target genes: TNFα, IL-6, and IL-10. Reactions were carried out using the PowerUp™ SYBR™ Green Master Mix (Cat. #A25742, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Each reaction was performed in a final volume of 10 µL. The composition of the reaction mixture is reported in **Table 1**.

Table 1: Composition of the RT-qPCR reaction mixture used for gene expression analysis. The final reaction volume was 10 µL and included PowerUp™ SYBR™ Green Master Mix, forward and reverse primers, cDNA template and nuclease-free water.

Reagent	Volume
PowerUp™ SYBR™ Green Master Mix	5 µL
PCR Forward and Reverse Primers	1 µL
Sterile purified water	3 µL
cDNA	1 µL

Amplification and fluorescence detection were executed using the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Cat. #1855196, Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycling conditions are shown in **Figure 6**.

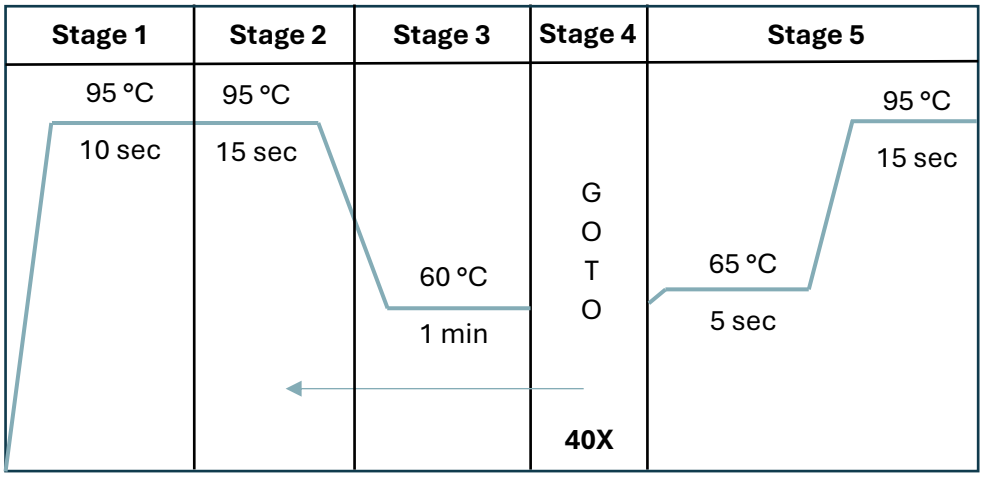

Figure 6: Thermal profile used for RT-qPCR analysis. The protocol consisted of an initial denaturation at 95°C, followed by 40 cycles of denaturation (95°C) and annealing/extension (60°C), and a final melt curve analysis.

All reactions were run in technical duplicate and biological triplicate. TBP (TATA-Box Binding Protein) served as the internal reference housekeeping gene for data normalization.

Gene expression levels were calculated using the ΔΔCt method. Results were expressed as relative quantity (RQ) compared with untreated controls.

The forward and reverse primer sequences (5' → 3') utilized for RT-qPCR amplification are summarized in **Table 2**.

Table 2: Primer sequences used for RT-qPCR amplification of the target genes. Forward and reverse primer sequences are shown in the 5'→3' direction for TBP, TNF-α, IL-10 and IL-6.

Gene	Forward Primer 5' → 3'	Reverse primer 5' → 3'
TBP	TGTATCCACAGTGAATCTTGGTTG	GGTTCGTGGCTCTCTTATCCTC
TNF-α	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGTCACTC
IL 10	GCCGAAAGAAGCTACCCAGTGT	GGTCCAAGTTCTTCAGCTCGTG
IL 6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG

4.10 Statistical Analysis

All experimental data obtained from both analyses were expressed as mean ± standard error of the mean (SEM) from triplicate samples. Statistical evaluations were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

To determine statistical significance between the control group and the different calcitriol concentration treatments, a one-way analysis of variance (ANOVA) followed by a post-hoc multiple comparison test was applied. The threshold for statistical significance was set at 0.05 (two-tailed).

5. RESULTS

5.1 Effect of calcitriol on U937 cell viability

To evaluate the effect of calcitriol treatment on U937 cells, cell viability was assessed after 24 h by Crystal Violet staining. As shown in **Figure 7**, similar values were observed among most treatment groups. The highest value was recorded in cells treated with 10 nM calcitriol, which showed a significant increase ($p < 0.01$) compared with the control group (CTRL). Representative images of Crystal Violet staining are shown in **Figure 8**.

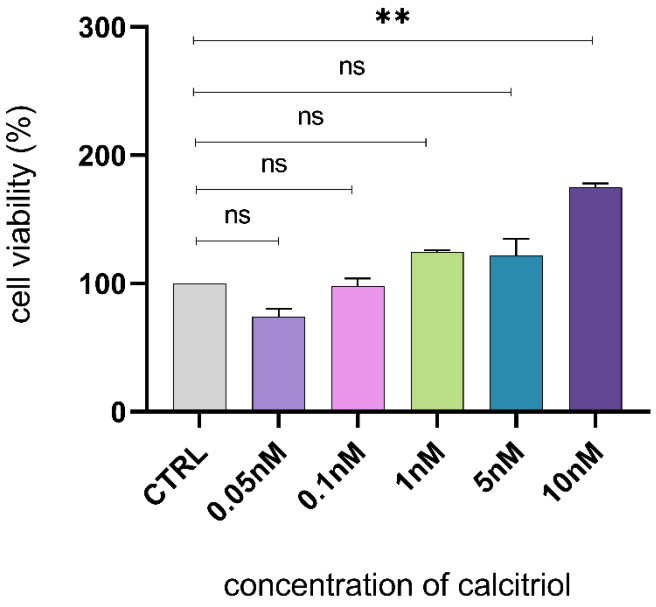

Figure 7: Analysis of cell viability assessed by Crystal Violet staining after 24 h of calcitriol treatment, normalized to untreated control (CTRL). ** $p < 0.01$, n= 2 biological replicates.

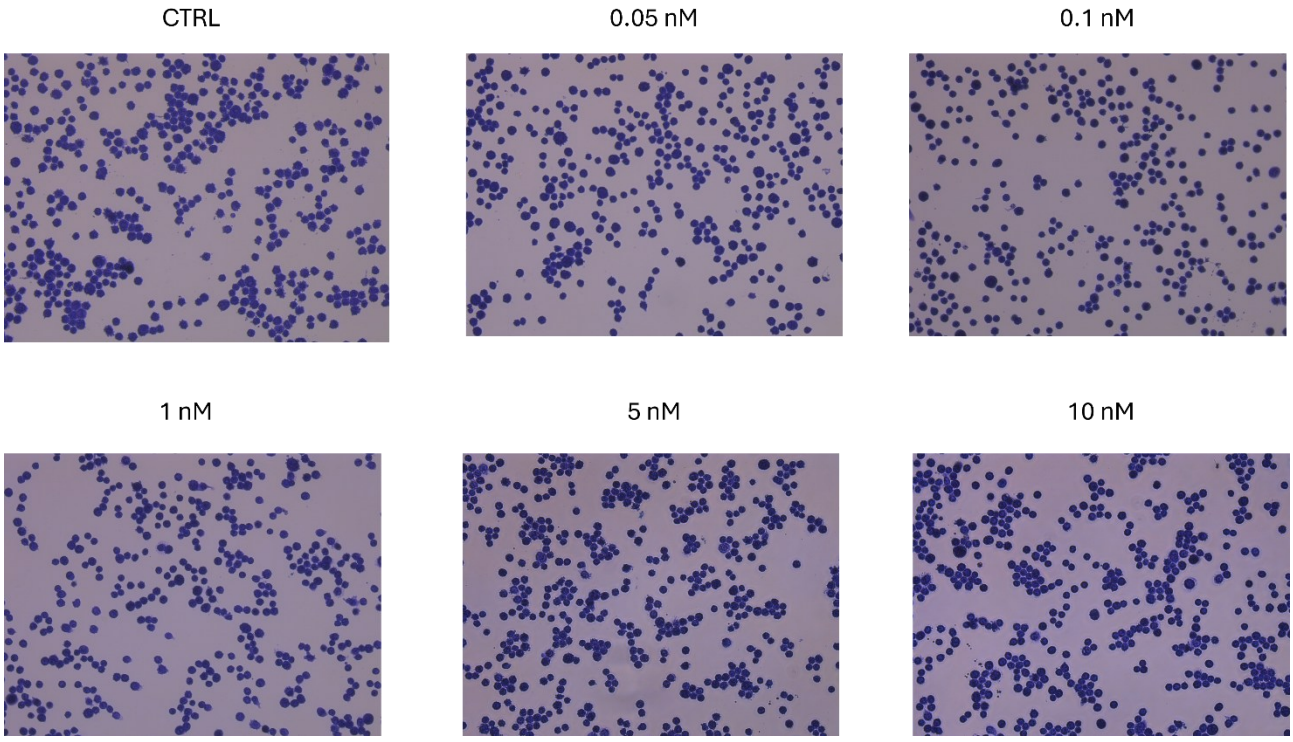

Figure 8: Representative images of U937 cells stained with Crystal Violet after 24 days of calcitriol treatment.

To evaluate the effect of prolonged calcitriol treatment on U937 cells, cell viability was assessed after 7 days by Crystal Violet staining. Cell viability values were similar to the untreated control (CTRL) at concentrations ranging from 0.05 nM to 5 nM (**Figure 9**). The highest value was observed in cells treated with 10 nM calcitriol, which showed a significant increase ($p < 0.0001$) compared with the untreated control. Representative images of Crystal Violet staining are shown in **Figure 10**. Cells displayed morphological features consistent with M1-like and M2-like phenotypes. Rounded cells resembling an M1-like morphology and elongated cells resembling an M2-like morphology could be observed.

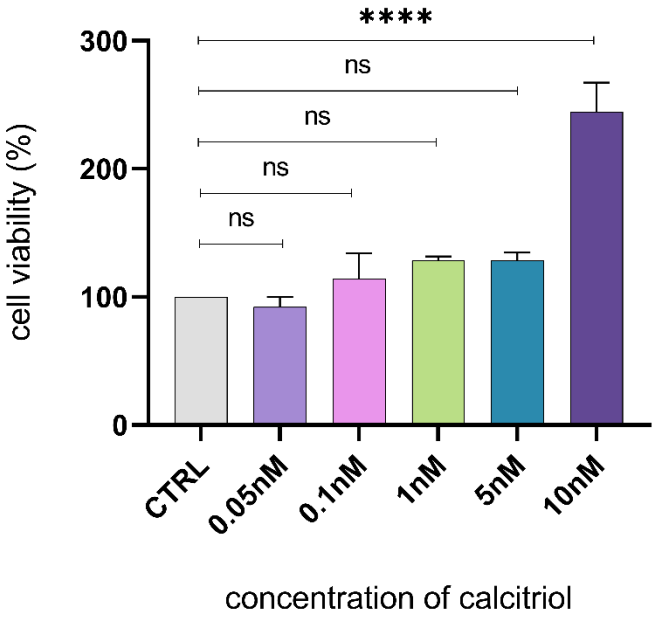

Figure 9: Analysis of cell viability assessed by Crystal Violet staining after 7 days of calcitriol treatment, normalized to untreated control (CTRL). **** $p < 0.0001$, n= 2 biological replicates.

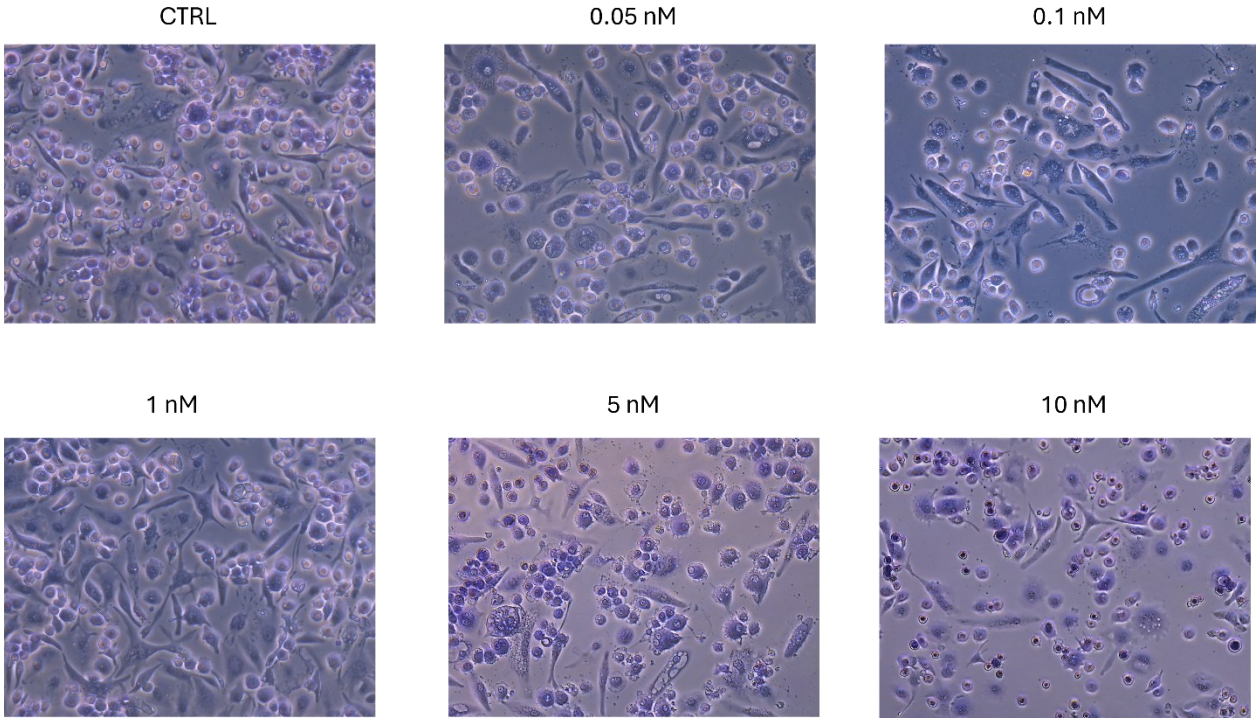

Figure 10: Representative images of U937 cells stained with Crystal Violet after 7 days of calcitriol treatment.

5.2 Effect of calcitriol on phagocytic activity

According to the results obtained by the Incucyte® SX5 system, phagocytic activity increased progressively over time in all groups. Treatment with calcitriol resulted in higher phagocytic index compared with untreated control (CTRL), with the most pronounced effect observed at 1 nM. Similar values were also observed in the 5 nM group. However, statistical analysis by one-way ANOVA showed that phagocytic activity differs significantly ($p < 0.0001$) in the 0.1 nM and 1 nM groups compared to untreated control, while no significant differences between the other groups were detected (**Figure 11**).

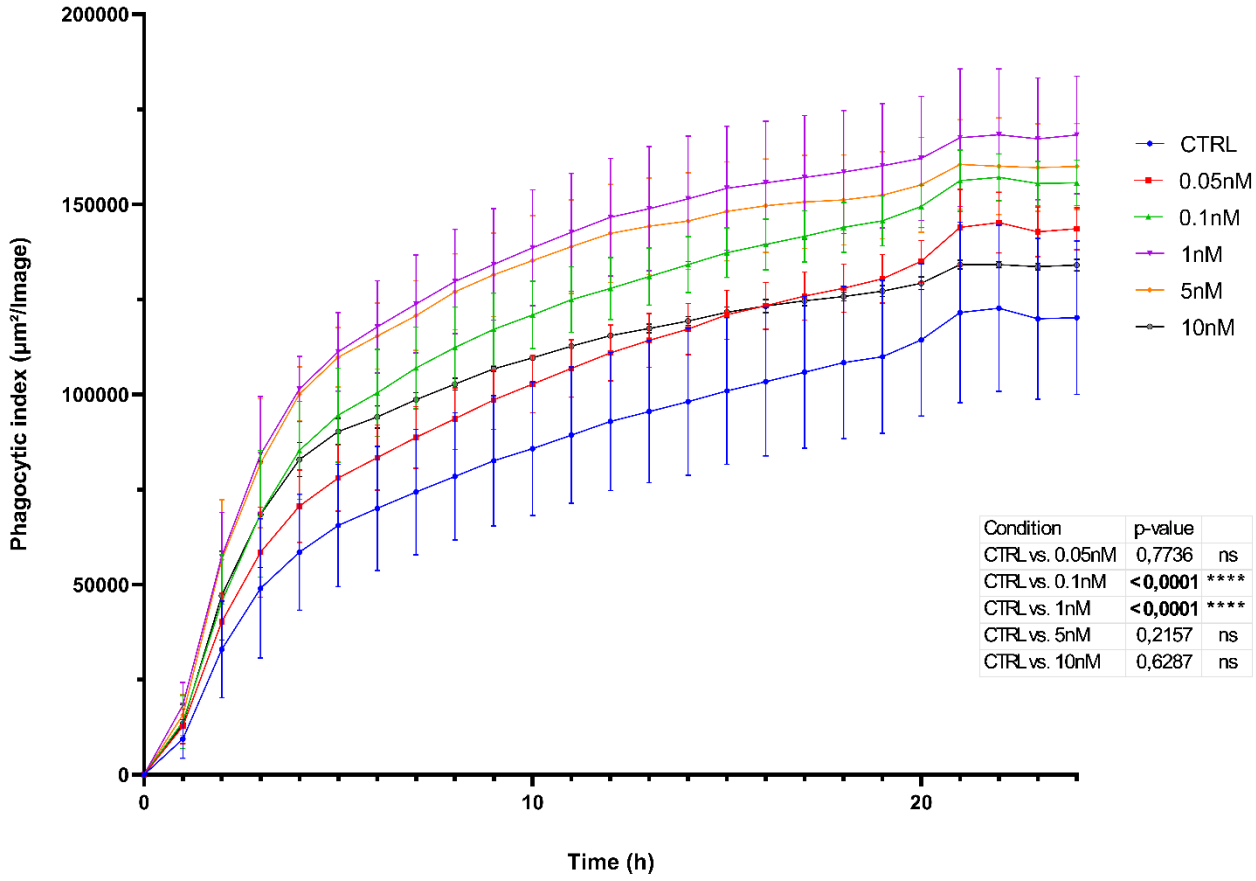

Figure 11: Phagocytic activity of U937 cells treated with different concentrations of calcitriol and monitored for 24 h. Data are presented as mean red fluorescence object area ± standard error of the mean (SEM), normalized to untreated control (CTRL). **** $p < 0.0001$, n= 3 biological replicates.

5.3 Effect of calcitriol on cytokine gene expression

To investigate whether, under polarizing conditions, calcitriol treatment may influence the expression of TNF-α, IL-6 and IL-10 in U937-derived macrophages, real time qPCR was performed. To mimic the physiological levels of calcitriol, a concentration of 0.1 nM was used.

As shown in **Figure 12**, TNF-α expression was significantly increased in M0 cells compared with the untreated control (CTRL). In contrast, a significant reduction in TNF-α expression was observed in both M1-like and M2-like cells. The lowest expression levels were detected in M2 macrophages.

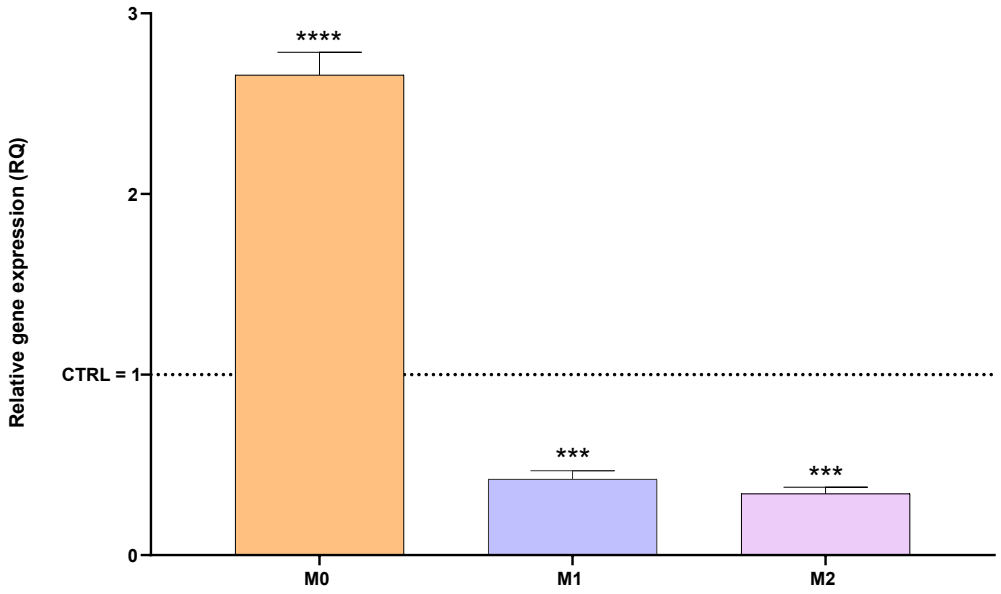

Figure 12: Gene expression of TNF-α gene in U937-derived macrophages under M0, M1, and M2 conditions after treatment with 0.1 nM calcitriol, compared with untreated controls (CTRL). Data are presented as relative quantity. *** $p < 0.001$ and ****$p < 0.0001$

Additionally, IL-6 expression increased in all experimental groups compared with the corresponding untreated controls (**Figure 13**). The highest expression levels were observed in M1-like cells, followed by M0 cells, whereas M2-like macrophages showed lower IL-6 expression. No statistically significant differences were detected among the experimental groups.

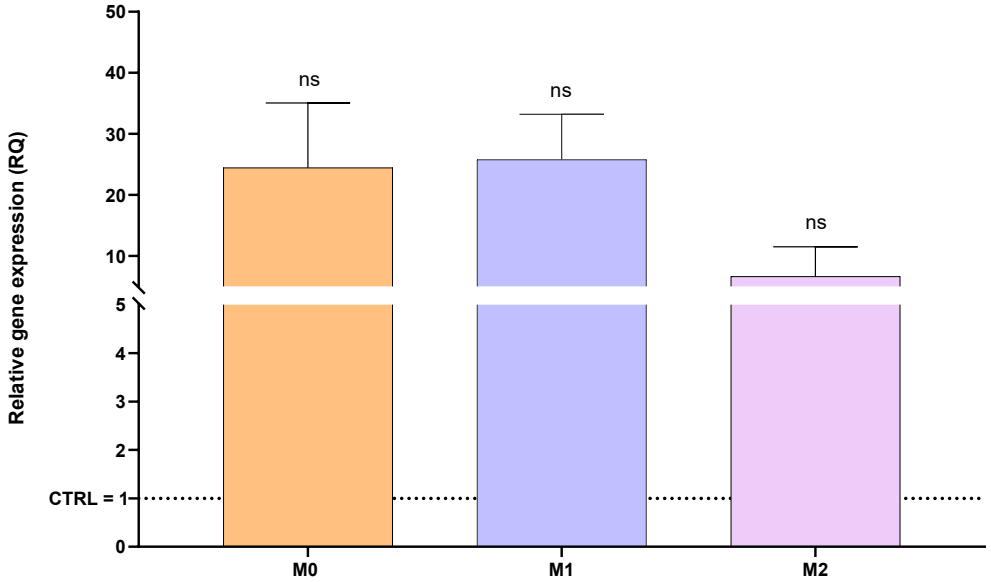

Figure 13: Gene expression of IL-6 in U937-derived macrophages under M0, M1 and M2 conditions following treatment with 0.1 nM calcitriol, compared with untreated controls (CTRL). Data are presented as relative quantity. ns= non-significant

Moreover, in **Figure 14** calcitriol treatment significantly increased IL-10 expression in M0 and M2-like cells compared with the corresponding untreated controls. The highest expression level was observed in M2-like macrophages, whereas M1-like cells showed only a slight increase in IL-10 expression.

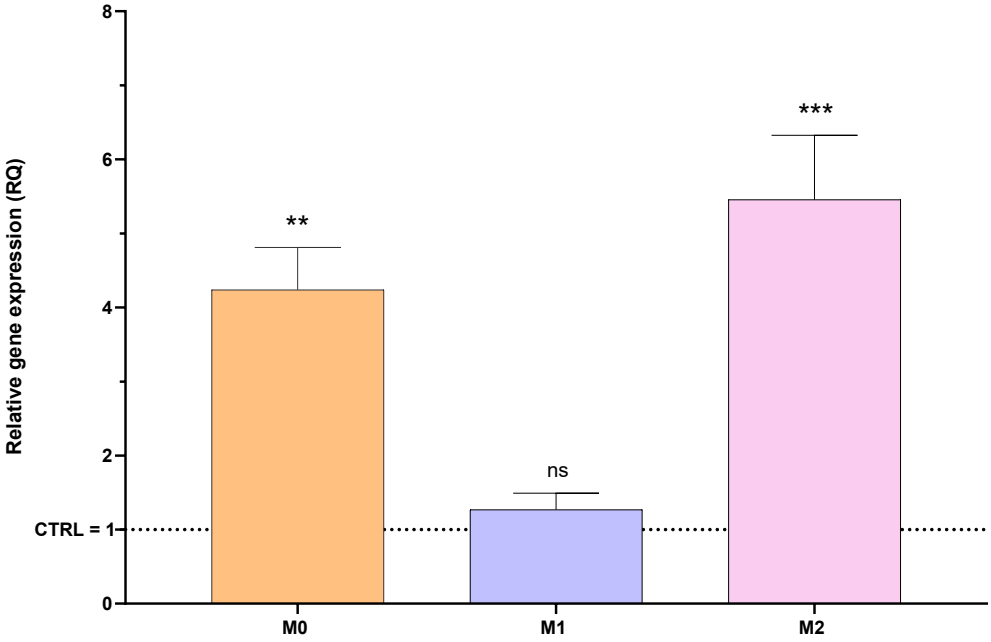

Figure 14: Gene expression of IL-10 in U937-derived macrophages under M0, M1 and M2 conditions following treatment with 0.1 nM calcitriol, compared with untreated controls (CTRL). Data are presented as relative quantity. ** $p < 0.01$, ***p <0.001, ns=non-significant.

6. DISCUSSION

The innate immune response represents the first line of defence against pathogens. It is characterised by its rapid and non-specific reaction to pathogens [2]. Macrophages represent a pivotal cell type within the innate immune system, responsible for maintaining tissue homeostasis and coordinating inflammatory responses [5]. Due to their remarkable plasticity, macrophages can acquire distinct functional phenotypes in response to environmental stimuli [13]. In addition, macrophages contribute to host defence through several essential functions, including phagocytosis, antigen presentation and efferocytosis. In particular, phagocytosis allows the recognition, uptake and degradation of pathogens, cellular debris and foreign particles, representing one of the most important mechanisms of innate immunity [9]. Classically activated M1 macrophages, induced by LPS and IFN-γ are characterised by the production of pro-inflammatory mediators, including TNF-α and IL-6. In contrast, activated M2 macrophages stimulated by IL-4 are associated with anti-inflammatory functions and the production of cytokines such as IL-10 [13]. In recent years, increasing attention has been directed toward the role of vitamin D in the regulation of immune responses. Beyond its effects on bone health, calcitriol has been shown to influence several functions of immune cells, including macrophage activation and cytokine production. Indeed, it has been reported that vitamin D enhances antimicrobial responses while promoting a shift toward a more anti-inflammatory phenotype [35]. In light of the current knowledge, this study aims to further explore the immunomodulatory role of vitamin D. In particular, the effects of calcitriol on U937-derived macrophages were evaluated by assessing cell viability, phagocytic activity and cytokine gene expression.

The Crystal Violet assay revealed a significant increase in cell viability following 10 nM calcitriol treatment after 24 hours and 7 days of exposure. These results suggest that calcitriol does not exert any cytotoxic effects on U937-derived macrophages under the experimental conditions used in this study. Furthermore, representative images after 7 days revealed the presence of cells displaying morphological features consistent with both M1-like and M2-like phenotypes, suggesting a potential role of calcitriol in the modulation of macrophage polarization *in vitro*. Similar observations have been reported in previous studies showing that vitamin D promotes macrophage differentiation and modulates macrophage functionality [40].

In the present study, calcitriol treatment enhanced the phagocytic activity of U937-derived macrophages. Specifically, cells treated with 0.1 nM and 1 nM calcitriol exhibited a significant

increase in the phagocytic index compared with the untreated control group. However, no significant differences were observed for the other concentrations tested. These results are consistent with previous studies demonstrating that vitamin D enhances macrophage phagocytic function[41].

Moreover, Dodd et al. reported that treatment with 1 nM and 10 nM calcitriol significantly increased bacterial ingestion in U937 cells [42]. Consistent with these findings, 1 nM calcitriol significantly enhanced phagocytic activity in the present study. However, unlike the results reported by Dodd et al., the increase observed at 10 nM did not reach statistical significance. This discrepancy may be related to differences in the experimental model, treatment duration, assay conditions and phagocytosis readout.

Based on the phagocytosis results, 0.1 nM calcitriol was selected for subsequent RT-qPCR analyses. In addition to significantly increasing phagocytic activity, this concentration is within the physiological range reported for circulating calcitriol levels [43], making it a biologically relevant condition for investigating cytokine gene expression.

The effects of calcitriol on TNF-α expression differs according to the macrophage polarization. In this study, treatment with 0.1 nM calcitriol significantly increased TNF-α expression in M0 macrophages, while a significant reduction was observed in both M1-like and M2-like macrophages. These observations are partially consistent with previous studies reporting that vitamin D suppresses the expression of pro-inflammatory cytokines, including TNF-α, in activated macrophages. In particular, Rafique et al. demonstrated that both calcidiol and calcitriol reduce TNF-α expression in human monocyte-derived macrophages, using calcitriol concentrations ranging from 0.05 nM to 10 nM [44]. The reduction of TNF-α expression observed in M1-like and M2-like macrophages is therefore consistent with the inhibitory effects of vitamin D metabolites reported in literature. However, vitamin D responses appear to vary according to the activation and polarization state of macrophages. Similarly, Stachowicz-Suhs et al. reported heterogeneous effects of the active vitamin D metabolite calcitriol on macrophage phenotypes [40]. This suggests that its immunomodulatory activity depends heavily on the cellular context.

In contrast to the effects observed for TNF-α, calcitriol treatment did not significantly affect IL-6 expression in any of the macrophage populations analysed. Although increased IL-6 expression was observed in M0, M1-like and M2-like macrophages compared with the corresponding untreated controls, no statistically significant differences were detected. The highest levels were observed in M1-like macrophages, which is consistent with their pro-inflammatory phenotype. These findings

differ from previous studies reporting that vitamin D suppresses the expression of pro-inflammatory cytokines, including IL-6, in macrophages. In particular, Rafique et al. demonstrated that both calcidiol and calcitriol reduce IL-6 expression in human macrophages [44]. This discrepancy may be related to differences in experimental conditions, including the macrophage model, treatment duration and stimulation protocol. Furthermore, studies have reported both suppressive and context-dependent effects of vitamin D on inflammatory cytokine expression [40]. The absence of significant changes in IL-6 expression observed in the present study may reflect the complex effects of vitamin D on macrophage inflammatory responses, which can vary according to macrophage phenotype and cellular context.

However, the increase in IL-10 expression observed following calcitriol treatment is consistent with the anti-inflammatory properties of vitamin D. The present study showed significantly higher levels of IL-10 expression in M0 and M2-like macrophages, with the highest levels observed in M2-like cells. Although IL-10 expression was also increased in M1-like macrophages compared with the untreated control, this difference was not statistically significant. Similar findings have been reported in previous studies demonstrating that active vitamin D promotes anti-inflammatory macrophage responses and enhances IL-10 production. In particular, Vaccaro et al. reported increased IL-10 expression following calcitriol treatment in macrophages, supporting the immunoregulatory role of vitamin D [45]. In addition, recent evidence has demonstrated the ability of calcitriol to promote anti-inflammatory pathways and cytokine profiles associated with macrophage regulation and tissue homeostasis [46]. Overall, these results suggest that calcitriol may potentially contribute to the development of a more anti-inflammatory macrophage profile.

In conclusion, the results obtained in this study suggest that calcitriol can modulate several functional aspects of U937-derived macrophages. Calcitriol treatment increased cell viability and enhanced phagocytic activity at specific concentrations, indicating a potential role in macrophage functionality. Furthermore, the cytokine expression analysis revealed a reduction of TNF-α expression in M1-like and M2-like macrophages together with an increase in IL-10 expression, supporting the immunomodulatory properties of vitamin D. However, some results, particularly those related to IL-6 expression and the increase of TNF-α observed in M0 macrophages, did not fully reflect the trends reported in the literature. These differences may be related to the experimental model and conditions used in the present study, highlighting the complexity of vitamin D-mediated regulation of macrophage responses. Overall, the findings suggest that calcitriol may

contribute to the modulation of macrophage phenotype and function, although further studies are required to better clarify the molecular mechanisms involved and to confirm these observations.

While the findings provide further insight into the immunomodulatory effects of calcitriol, several limitations should be taken into consideration. First, the use of a single *in vitro* macrophage model, may not fully reproduce the complexity of macrophage responses *in vivo*. In addition, cytokine expression was evaluated only at the gene level and the corresponding protein production was not assessed. Another limitation is that RT-qPCR analyses were performed using a single calcitriol concentration (0.1 nM), selected on the basis of the phagocytosis results and its physiological relevance. Therefore, potential dose-dependent effects of calcitriol on cytokine expression could not be evaluated.

Future studies could investigate the effects of a broader range of calcitriol concentrations on macrophage cytokine expression to better characterize possible dose-dependent responses and identify the concentrations associated with the strongest immunomodulatory effects. In addition, further studies could explore the molecular mechanisms underlying the effects of calcitriol on macrophage function by evaluating proteins involved in inflammatory signalling pathways and macrophage polarization. The assessment of cytokine secretion at the protein level would also help to determine whether the observed changes in gene expression are reflected in functional responses. Finally, validation of these findings in primary human macrophages and more physiologically relevant *ex vivo* models would provide further insight into the immunomodulatory role of calcitriol.

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