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Mechanistic crosstalk of extracellular calcium-mediated regulation of maturation and plasticity in human monocytes

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ABSTRACT

Innate immune cells play a pivotal role in controlling tissue repair and rejection after biomaterial implantation. Calcium supplementation regulates cellular responses and alter the pathophysiology of various diseases. A series of macrophage activations through differential plasticity has been observed after cell-to-material interactions. We investigated the role of calcium supplementation in controlling macrophage phenotypes in pro-inflammatory and pre-reparative states. Oxidative defence and mitochondria involvement in cellular plasticity and the sequential M0 to M1 and M1 to M2 transitions were observed after calcium supplementation. This study describes the molecular mechanism of reactive oxygen species and drives the interconnected cellular plasticity of macrophages in the presence of calcium. Gene expression, and immunostaining, revealed a relationship between MHC class II maturation and cellular plasticity. This study elucidated the role of controlled calcium supplementation under various conditions. These findings underscore the molecular mechanism of calcium-mediated immune induction and its favourable use in different calcium-containing biomaterials., essential for tissue regeneration.

1. Introduction

Plasticity and functional polarization are hallmarks of the mononuclear phagocytic system in response to tissue microenvironmental stimuli which based on microenvironmental cues [1]. In presence of interferon (IFN)-γ and granulocyte-macrophage colony-stimulating factor, monocytes differentiate into M_1 macrophages, which stimulate the production of interleukin (IL)-1β, IL-12, tumor necrosis factor (TNF)-α, and superoxide anions [2]. In the presence of adenosine, M_1 macrophages tend to produce the IL-4-receptor, which direct them toward an M_2-like phenotype [3]. Further they tend to produce IL-10, IL-1α, transforming growth factor (TGF)-β, and arginase-1(Arg1), and suppress inflammation [4].

In the present study, we have recapitulated the localized inflammatory tissue microenvironment using immune response-based cell phenotype. Various biomaterials have been investigated for controlled calcium release for macrophage plasticity [5]. A previous study elaborates the role of monocyte calcium influx and efflux and their role in cytoplasmic ionized calcium exchange to alter the cellular plasticity in monocytes. The calcium reaching microenvironment acts as a second messenger for macrophage plasticity in the absence of voltage-gated channels [6]. The mitochondrial calcium uniporter, a mitochondrial inner membrane protein, contributes to calcium entry into the matrix; however, its correlation with phagolysosome maturation and plasticity remains unclear [7]. In this study, we evaluated the correlation between major histocompatibility complex (MHC) class II maturation and macrophage plasticity in optimal calcium concentrations.

Thus, we aimed to understand inflammation-relevant changes in THP-1 plasticity following exposure to calcium ions in an in vitro environment. We determined the optimum calcium concentration required to maintain cellular plasticity without growth factors and quantified the oxidative defence of THP-1 cells and its possible correlation with plasticity in presence of calcium. We investigated whether MHC class II maturation is positively correlated with macrophage plasticity in the presence of extracellular calcium. We used pH-dependent and antibody-mediated lysosomal and endosomal trafficking [8] and MHC class II maturation o identify its effect on downstream maturation.

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2. Materials and methods

2.1. Materials

RPMI-1640 medium (Gibco), RPMI-1640 medium-phenol red-free (Gibco), penicillin-streptomycin-glutamine (PSG) (Gibco), fetal bovine serum (FBS)(HiMedia), PMA (Sigma-Aldrich), IL-4 (HiMedia),IL-10 (HiMedia), calcium chloride (Merck), thiazolyl blue tetrazolium bromide (MTT)(Sigma-Aldrich), Assay kit ((TBARS,GST, antioxidant, nitric oxide (NO))(Himedia) LysoTracker Red D99 (50 nM; Molecular Probes), ER-Tracker Red (Molecular Probes), DAPI (Sigma-Aldrich), Mito-ID Green Detection Kit (ENZO), lysosome-associated membrane proteins (LAMP-2; Origene), Fura-2 AM, Ras-related protein Rab-11(ST John), Alexa Fluor 488 and 633 (Invitrogen), RNeasy Mini Kit (Qiagen), cDNA synthesis kit (Thermo Fisher Scientific), Primers (GAPDH, CD 14, CD 36, CD 68, IL 4, IL 10) from Qiagen. THP-1 cell line has been procured from National Centre for Cell Science (NCCS), Pune.

2.2. Cell culture

THP-1 monocytes were cultured in RPMI-1640 medium with 1% PSG and 10% FBS. The cells were kept at 37 °C in an incubator [9].

2.2.1. Differentiation of THP-1 monocytes into THP-1 M_0 macrophage

THP-1 monocytes were differentiated into M_0 macrophages using 200 nM PMA with 1% PSG and 10% FBS. The cells were incubated at 37 °C. After 7 days, THP-1 monocytes were expected to differentiate into THP-1 M_0 macrophages [10].

2.2.2. Differentiation of THP-1 M_0 macrophages into THP-1 M_2 macrophage

THP-1 M_0 macrophages were cultured using IL-4 (200 ng/μL), IL-10 (200 ng/μL) in 1% PSG, 10% FBS, and RPMI-1640 medium. The cells were incubated at 37 °C. After five days, THP-1 M_0 macrophages were differentiated into THP-1 M_2 macrophages [11].

2.3. Assessment of metabolic activity

THP-1 monocytes were cultured for 1, 2, and 3 days at 0–500 μg/ml calcium ion concentration. The IC50 for THP-1 monocyte proliferation in different calcium ion concentrations was calculated using the MTT assay for 26hrs. The reduction of MTT was measured as absorbance at the excitation wavelength of 570 nm and emission wavelength of 595 nm using a microplate reader [12].

2.4. Identification of oxidative defence mechanism after calcium supplementation

To analyse chemokine release kinetics, the media of THP-1 monocytes, M_0 and M_2 macrophages were collected from the control and in the presence of calcium on days 1, 3, and 7.

2.4.1. Estimation of released antioxidants, NO, lipid peroxidation, glutathione S-transferases

The media of THP-1 monocytes, M_0 and M_2 macrophages with calcium ions on days 1, 3, and 7 were compared with those of untreated cells. FRAP values were obtained at absorbance 593 nm in the test reaction mixtures containing ferrous ions at known concentrations to determine released antioxidants, at 560 nm for NO [13], 532 nm for lipid peroxidation [14] and at 340 nm [15] for glutathione S-transferases.

2.5. Live-cell microscopy to identify mitochondrial biogenesis

THP-1 monocytes, and M_0, M_2 macrophages were seeded at a density of 1×10^6 cells in collagen-coated Petri dishes and incubated for 24 h. Subsequently, the cells were incubated with IC50 of calcium for 7 days and compared with the untreated cells. Cells were incubated at 37 °C. for at least 2 h with 2 μl/ml Mito ID Green and stained with Hoechst 33342 for 15 min which can be detected using a fluorescein filter set (excitation, 485 nm: emission, 530 nm) [16].

2.6. Live-cell microscopy to identify MHC class II maturation

2.6.1. pH-dependent lysosomal and ER trafficking

THP-1 monocytes, M_0 and M_2 macrophages were incubated with calcium for 1, 3, or 7 days. At this time point, cells were incubated at 37 °C for 2 h with 50 nM Lysotracker Red D99 and ER-Tracker Red in phenol red-free RPMI 1640 supplemented with FBS and PSG, stained with Hoechst 33342 for 15 min before the experiment.

2.6.2. Identification of lysosomal (LAMP-2) and endosomal (RAB-11) cell surface marker

THP-1 monocytes, THP-1 M_0 macrophages, and THP-1 M_2 macrophages were used as controls and day 7 samples. A blocking solution was applied overnight at 4 °C. The samples were incubated overnight at 4 °C with mouse anti-human Rab-11 (1:200) and rabbit anti-human LAMP-2 (1:200) primary antibodies. Secondary antibodies labelled with Alexa Fluor 488 and546 were used, and DAPI was used to for nucleus staining.

2.6.3. Fura-2 AM staining to identify intracellular calcium

The cells were incubated with calcium for 7 days and processed for calcium imaging. The cells are washed properly with PBS and incubated with 5 μM Fura-2 AM for 2 h in 37 °C in dark. Cells were washed by PBS and incubated with 5 μL of rhodamine phalloidin in PBS with 1% BSA for 1 h 1 μL Hoechst 33258 for 15 min was added.

2.7. Gene expression analysis

Cell lysates were collected on days 14 and 7 for untreated cells, and on days 1, 3, and 7 for calcium-treated cells. The total extracted RNA was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit, and SYBR Green Master Mix and a Rotor gene Q thermocycler was used for RT-PCR [17]. The surface markers studied were CD-14, CD-36, CD-68, IL-4, and IL-10.

2.8. Statistical analysis

GraphPad Prism 7 software (San Diego, USA) was used for statistical analysis. One-way ANOVA followed by Bonferroni multiple comparison test was used for oxidative defence mechanism and gene expression analysis.

3. Results

3.1. Effect of calcium ions on the metabolism of THP-1 monocytes

There was an increase in the cell metabolic activity from day 1–2 at all calcium ion concentrations (Supplementary Fig. 1). Cell metabolic activity decreased on day 3, except for 450 μM, 4 mM, and 4.5 mM ion concentrations. Based on this observation, we calculated the IC50 values of calcium ions at all-time points. IC50 for day 1 was 140 μM, which decreased to 130 μM and 120 μM on days 2 and 3.

3.2. Release profile of oxidative stress marker

The release of NO increased by 2.21 folds ($p < 0.0001$) in presence of calcium ions at day 1 in THP-1 monocytes (Fig. 1A) [29]. An elevated release of 3.46 ($p < 0.0001$) was observed at day 7. The release of NO from THP-1 M_0 macrophages was increased by 1.75 ($p < 0.0001$) and 1.39 ($p < 0.0001$) in the presence of calcium ions at day 1 and day 7, respectively. The control set of THP-1 M_2 macrophages showed a decreased release of 0.99 folds ($p < 0.0001$) in presence of calcium ions at day 1. The release of THP-1 M_2 macrophages was increased at day 7 by 1.38 folds ($p < 0.0001$) in the presence of calcium ions.

The release of antioxidants subsided by 0.42 folds (p-not significant) in presence of calcium ions for THP-1 monocytes at day 1 (Fig. 1B). At day 7, in the presence of calcium ions, THP-1 monocytes showed increase of 2.7 folds ($p < 0.0001$) compared to without calcium ions. The release of antioxidants from THP-1 M_0 macrophages increased by 1.89 ($p < 0.0001$) and 1.55 folds ($p < 0.0001$) in the presence of calcium ions at day 1 and day 7, respectively. THP-1 M_2 macrophages showed decreased release of antioxidants by 0.54 folds ($p < 0.0001$) and 0.62 folds ($p < 0.001$) in presence of calcium ions at day 1 and 7.

The release of lipid peroxides and their downstream markers in THP-1 monocytes, THP-1 M_0 and M_2 macrophages was not significantly different in the presence and absence of calcium (Fig. 1C). The negligible expression of TBARS and GST indicated no cellular membrane damage under any conditions upon exposure to calcium ions, as it is a direct marker of lipid peroxidation (Fig. 1D).

3.3. Effect of calcium ions on mitochondrial biogenesis

Controlled regulation presents a different class of macrophage phenotype (Fig. 2A and D). The smallest network size reduced mitochondrial membrane potential by incubation of M0 macrophages with calcium ions, and the shortest branch was observed (Fig. 2B and D). This indicates the predominance of the M_1 subpopulation. In contrast, in M_2 macrophages on day 7, a more energetic mitochondrial mass was evident by bright green fluorescence (Fig. 2C and D). Active mitochondria, with controlled glycolysis represent the M_2 population.

3.4. Effect on cellular plasticity and maturation

3.4.1. pH dependent intracellular trafficking within THP-1

M_2 macrophages exhibited much larger lysosomes than M_1 macrophages in the presence of calcium ions. M1 macrophages showed ovoid and spindle shapes and tubular morphology, whereas we observed a round morphology in M_2 macrophages. In the presence of calcium, M_2 macrophages showed a higher signal than M_1 macrophages in the ER and lysosomal staining. Cells cultured in extracellular calcium showed modifications by increasing signal intensity and changes in morphology (Fig. 3A and A). This change in the morphology of THP-1 monocytes from perfectly round cells indicates a shift in plasticity. THP-1 M_0 macrophages transitioned to multinucleated cell morphology (Figs. 3B and 4B). THP-1 M_2 macrophages retained the differentiated multinucleated, round macrophage morphology and enhanced

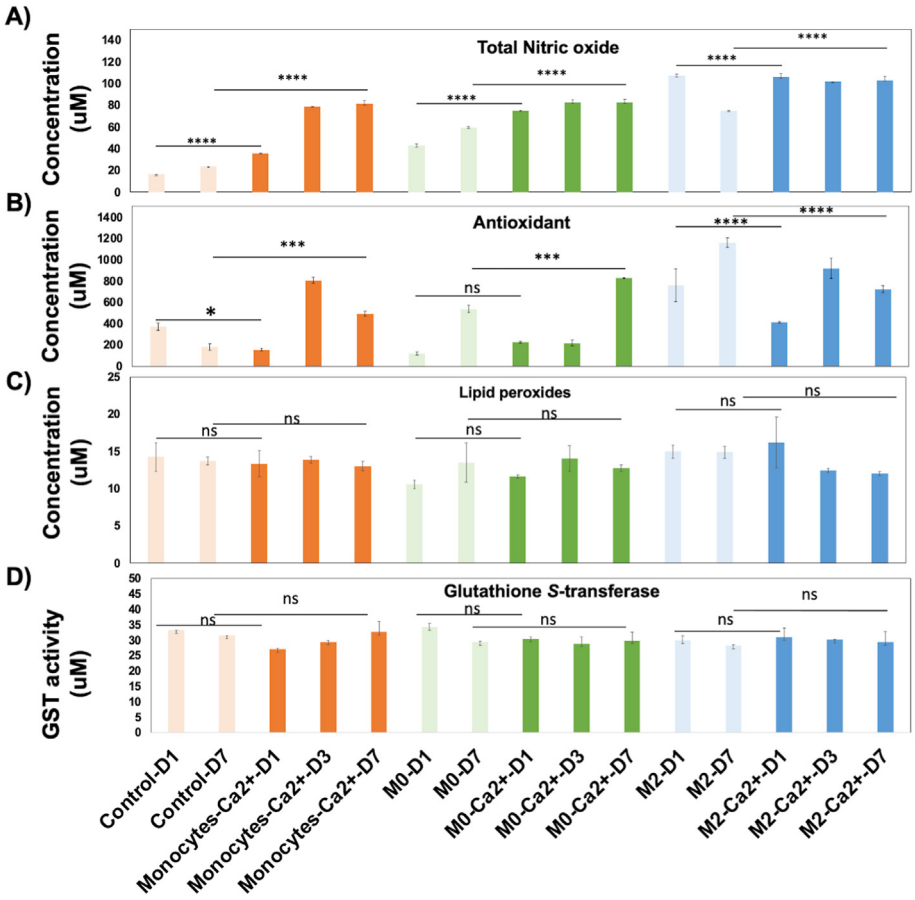

Fig. 1. Release profile of Nitric oxide, Antioxidants, Lipid peroxide and GST activity from THP-1 Monocytes, THP-1 M_0 Macrophages and THP-1 M_2 Macrophages after incubation in the presence of calcium ions for 1, 3 and 7 days.

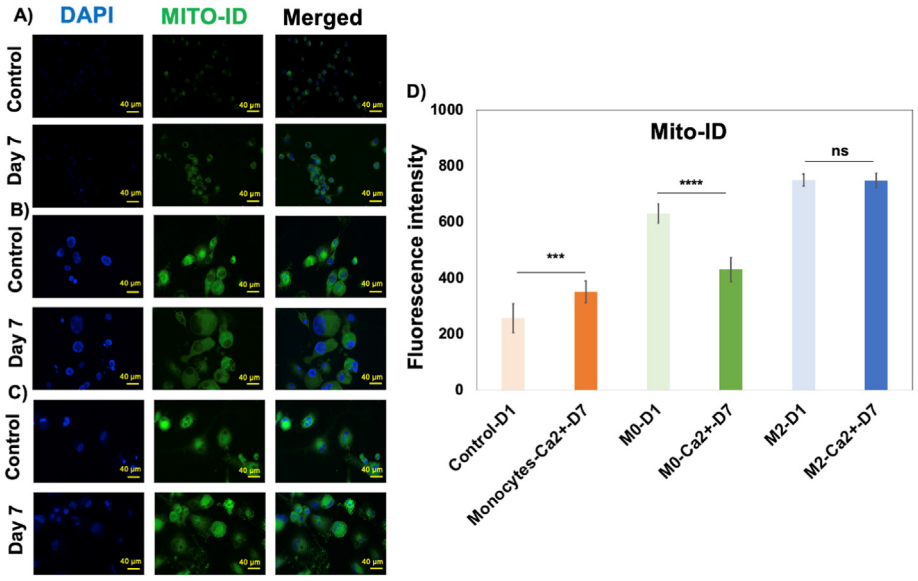

Fig. 2. Mitochondrial biogenesis in THP-1 cells in absence and presence of extracellular calcium A) THP-1 Monocytes B) THP-1 M_0 Macrophages C) THP-1 M_2 Macrophages D) Quantified fluorescence intensity.

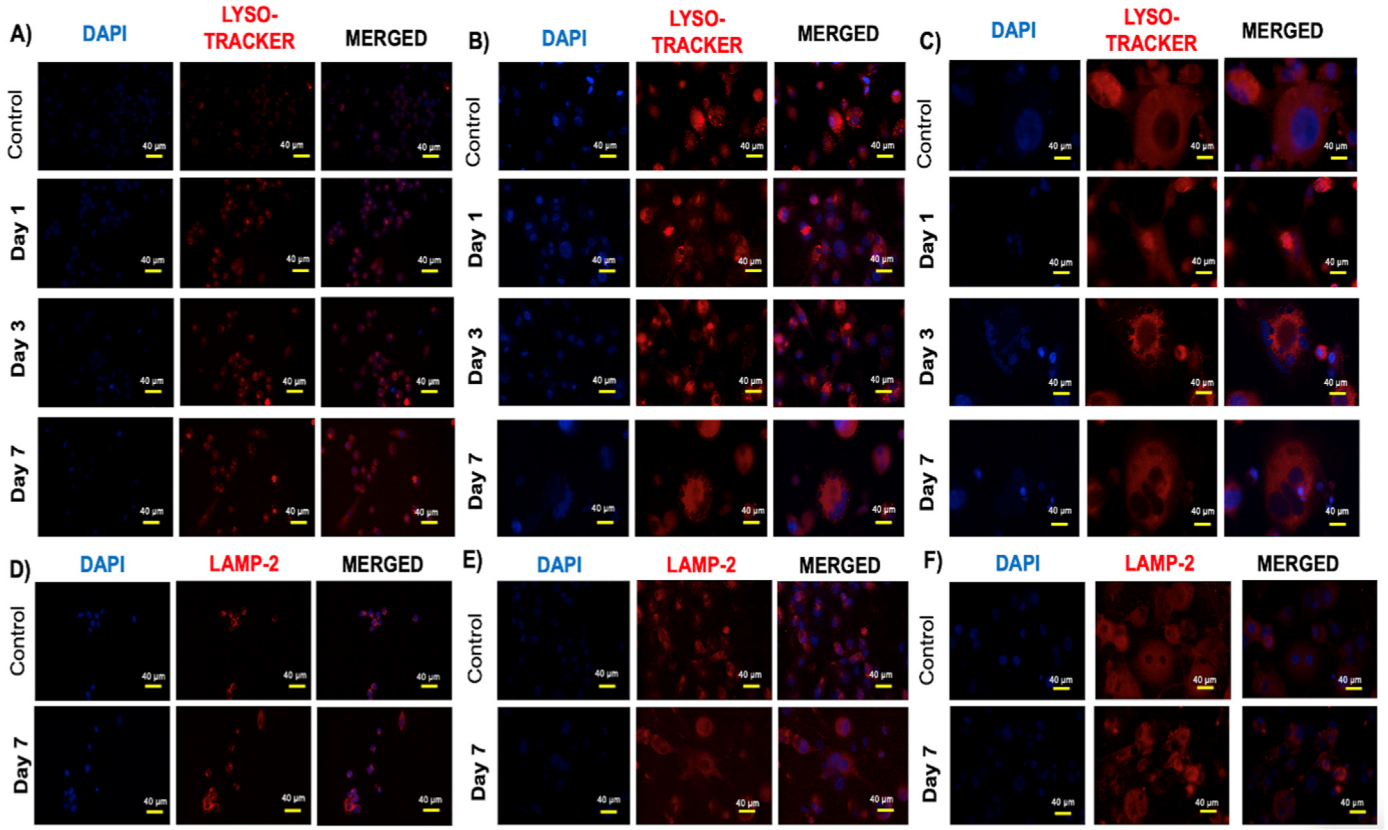

Fig. 3. Lysosomal trafficking A) Lyso-Tracker in THP-1 Monocytes B) Lyso-Tracker in THP-1 M_0 Macrophages C) Lysotracker in THP-1 M_2 Macrophages D) LAMP-2 in THP-1 Monocytes E) LAMP-2 in THP-1 M_0 Macrophages F) LAMP-2 in THP-1 M_2 Macrophages.

lysosome volume and size, even in the absence of IL-4 and IL-10 (Figs. 3C and 4C).

3.4.2. Surface marker of intravesicular compartmental proteins

Calcium ions effectively increased RAB-11 expression in M_0 macrophages, and maximum signal intensity was observed in M_2 macrophages. However, very weak expression was observed in the monocyte group. This indicated that calcium ions can interact with intracellular compartments in THP-1 cells and increase their surface marker levels (Fig. 4D, E, and F). LAMP-2 recruits phagosomes to

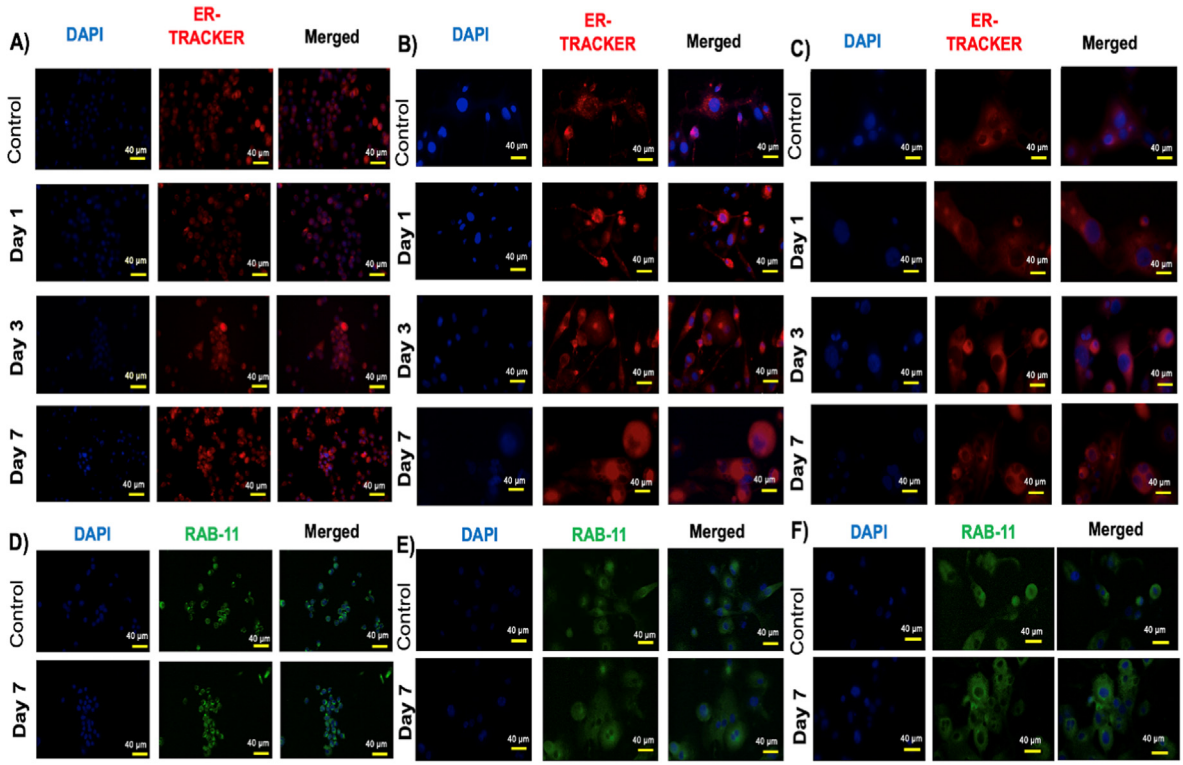

Fig. 4. Endosomal trafficking A) ER-Tracker in THP-1 Monocytes B) ER-Tracker in THP-1 M_0 Macrophages C) ER-Tracker in THP-1 M_2 Macrophages D) RAB-11 in THP-1 Monocytes E) RAB-11 in THP-1 M_0 Macrophages F) RAB-11 in THP-1 M_2 Macrophages.

selectively fuse with lysosomal vesicles and initiate phagolysosome biogenesis [18]. The increased LAMP-2 signal from M_2 macrophages confirmed the effect of calcium ions on THP-1 cells with increased lysosomal content and maturation. (Fig. 3 D, E, and F).

3.4.3. *Fura-2 AM staining for calcium*

Here we have observed the calcium uptake by Fura-2 AM staining. The calcium uptake in THP-1 monocytes is very lees at day 1 (Fig. 5A). Significant increase in calcium uptake was observed in M0 and M2 macrophage when cultured up to 7 days in presences of calcium (Fig. 5B and C). Mobilization of sustained intracellular calcium has been shown to maintain the cellular plasticity of M0 and M2 macrophage up to 7 days in culture conditions without growth factor.

3.5. *Gene expression*

CD-14 is a surface marker specific to monocytes, downregulated upon changes in THP-1 plasticity [19]. There was a 1.55 folds increase in the expression of CD-14 surface markers at day 1 in the presence of calcium ions for THP-1 monocytes (Fig. 6A). However, at day 7, there was a decrease of 0.24 folds. The expression of CD-14 in THP-1 M_0 macrophages increased by 1.5 folds at day 1 in the presence of calcium ions for but decreased by 0.06 folds at day 7 in the presence of calcium ions. THP-1 M_2 macrophages showed decreased expression from day 1 to day 7 by 0.56 and 0.27 folds, respectively in the presence of calcium ions.

There was 1.45 folds increase in the expression of CD-36 surface markers at day 1 in the presence of calcium ions for THP-1 monocytes (Fig. 6B), with further increase of 2.12 folds at day 7. Expression of CD-36 in THP-1 M_0 macrophages increased by 1.05 folds at day 1 in the presence of calcium ions for but decreased by 0.78 folds at day 7. The control set of THP-1 M_0 macrophages transformed into a monocyte-like morphology, whereas in the presence of calcium ions, THP-1 M_0 macrophages differentiated into an M_1 macrophage-like morphology. THP-1 M_2 macrophages also showed a decrease in CD-36 in the absence of interleukins (IL4 + IL-10). THP-1 M_2 macrophages with calcium ions showed a continuous decline in expression from day 1 by 0.83 folds, which increased by 1.79 folds at day 7. For the CD-68 surface marker, there was a 0.46-fold decrease at day 1 in the presence of calcium ions for THP-1 monocytes (Fig. 6C). However, there was an increase of 8 folds at day 7 in the presence of calcium ions. Expression of CD-68 in THP-1 M_0 macrophages decreased by 0.85 and 0.59-fold at day 1 and day 7 in the presence of calcium ions. The control set of THP-1 M_0 macrophages was transformed into a monocyte-like morphology. The THP-1 M_2 macrophage control set showed a negative expression of CD-68. THP-1 M_2 macrophages showed a continuous expression from day 1 to day 7 of 4.56folds in the presence of calcium ions.

There was no expression of IL-4 surface markers in THP-1 monocytes at day 1 in both conditions (Fig. 6D). Whereas upregulation of 3.48 folds upregulation was observed on day 7 in presence of calcium. The expression of IL-4 in THP-1 M_0 macrophages increased by 1.46 folds from the control group at day which further increased by 2.60 folds at day 7 in the presence of calcium ions. In the THP-1 M_2 macrophage, there was a decrease in IL-4 by 0.89 folds at day 1 in the presence of calcium ions. THP-1 M_2 macrophages showed 1.79 folds increase at day 7 in the presence of calcium ions for. There was no or no expression of the IL-10 surface marker in control THP-1 monocytes (Fig. 6E). There was increase of 1.14–2.56 folds from day 1 to day 7 in the presence of calcium ions. The expression of IL-10 in THP-1 M_0 macrophages decreased by 1.02 folds at day 1 in the presence of calcium ions. Similarly, there was an increase of 1.81 folds from day 1–7 in the presence of calcium ions. The THP-1 M_2 macrophage showed a decrease of 0.94

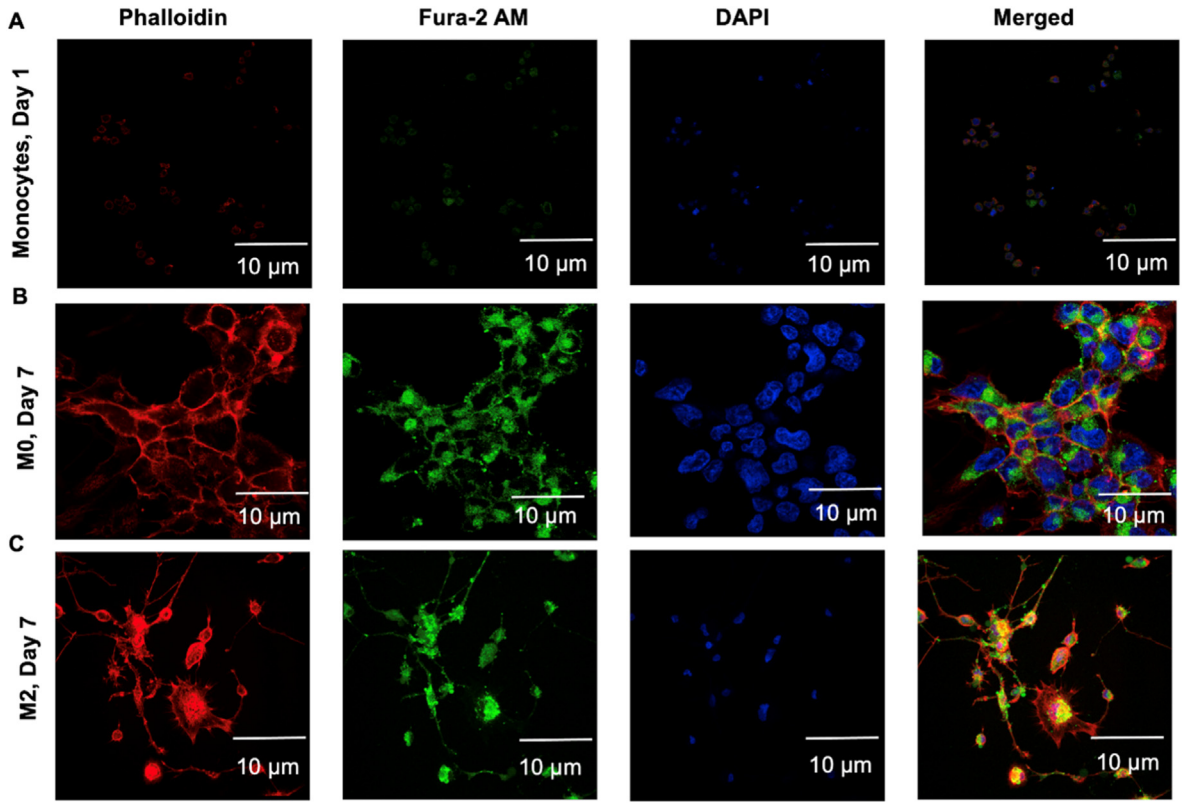

Fig. 5. Calcium uptake in THP-1 cells was observed A) THP-1 Monocytes (1 Day) B) THP-1 M_0 Macrophages (7 Day) C) THP-1 M_2 Macrophages (7 Day) at 63× magnification.

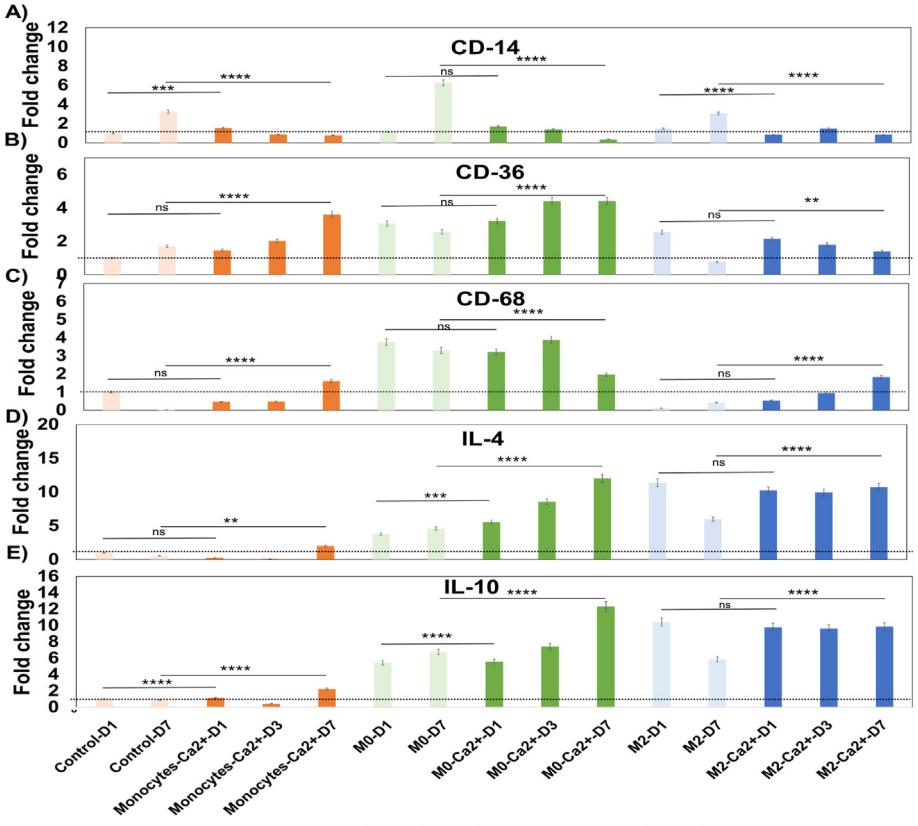

Fig. 6. Gene expression for THP-1 Monocytes, THP-1 M_0 Macrophages and THP-1 M_2 Macrophages for the different cell surface markers. 6 A) CD14, 6 B) CD36, 6 C) CD68, 6 D) IL-4, 6 E) IL-10.

folds in the expression of IL-10 at day 1 in the presence of calcium ions. THP-1 M_2 macrophages at day 7 showed increase of 1.69 at day 7.

4. Discussion

Calcium phosphate has been widely used as a biomaterial for regeneration. However, their role in inflammation and regeneration is always due to the differential regulation of macrophage plasticity [20]. The basic understanding of calcium-mediated regulation of macrophage cell phenotype post-implantation is still unclear. The role of calcium ions in immune activation has encouraged understanding the underlying mechanisms. Therefore, in this study, we aimed to understand changes in THP-1 cell morphology and plasticity in the presence of different calcium ions in the extracellular microenvironment.

Increased calcium mobilization from the extracellular space to the cytosol may induce apoptosis by augmenting Ca^{2+}-dependent protease activity [21]. We identified the optimum IC-50 value for extracellular calcium ion concentration (15.32 μg/ml), which is necessary to change plasticity while maintaining cell viability. This concentration does not overload calcium-sensing receptors, triggering cell death, but effectively changes intermediate plasticity [22].

Arginine metabolism is a central dichotomy via NO synthase or arginase that controls macrophage plasticity [23]. In macrophages, arginine is metabolized via two major pathways. It is metabolized by nitric oxide synthase to NO, citrulline, or ornithine/urea by arginase [24]. Several studies have identified arginine as a biological precursor to identify activated macrophages based on their nitrite and nitrate release patterns [25]. After calcium supplementation of M_2 macrophages, it acts as an anti-inflammatory agent via the diversion of arginine. However, in M_0 macrophages, calcium supplementation led to a change in plasticity towards the M_1 phenotype; therefore, we observed variable levels of NO.

To the best of our knowledge, few studies have elaborated on its intermediate role towards the change in plasticity and involvement in oxidative defence after calcium supplementation. It has been previously observed that human monocytes increase ROS levels during 1 alpha, 25-dihydroxy vitamin D-mediated differentiation [26]. Calcium ions stimulate THP-1 cells to activate the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase present in the plasma membrane to produce antioxidants [27]. Calcium supplementation of M_0 macrophages consistently increased antioxidant levels, indicating sustained polarization towards the M_1 phenotype. We hypothesized that M_2 macrophages stimulate increased arginase-1 activity to maintain reduced antioxidant and stable NO levels [28]. To determine the toxic effect of calcium supplementation on THP-1 cells, we estimated lipid peroxidation and GST levels and found a possible correlation between NO and ROS. However, M_0 and M_2 macrophages showed low levels of TBARS and GST, which indicates that the IC50 doses used for THP-1 are non-toxic but favourably support alteration of plasticity. Here, we have summarized the mechanistic insight of calcium supplementation in different plasticity of human macrophages, which reveals the role of the cellular antioxidant pathway involving antioxidant and nitric oxide release patterns.

Hence, it is explicit that M_1 macrophages utilize glycolysis, de novo fatty acid synthesis, and the pentose phosphate pathway to maintain their pro-inflammatory state [28]. However, M_2 macrophages inhibited oxidative phosphorylation and fatty acid oxidation, supporting this anti-inflammatory subtype. After identifying the characteristic metabolic signature from the biochemical assay, we engaged in mitochondria-mediated live-cell metabolic imaging to determine the role of calcium in manipulating macrophage plasticity [29]. In all activation states, M_1 macrophages exhibited a diverse mitochondrial morphology, including short branches and tubular morphology, with reduced mitochondrial membrane potential in the control group. M_2 macrophages showed a different pattern by shifting the M_1/M_2 ratio towards the M_2 subpopulation. They offer a stable high mitochondrial membrane potential with higher interconnections, larger mitochondrial networks, and highly energized mitochondria [30]. Our study aimed to correlate mitochondrial biogenesis and metabolism with our preceding data on the oxidative defence machinery [31].

LAMP-2 has been reported to be involved in maturation and phagocytosis. Normally, the lysosomal compartment is acidic, but the M_1 macrophage lysosome lacks significant proton-pumping activity in M_2 macrophages [32]. It has already been established that high NADPH levels are responsible for maintaining a neutral (7.55 ± 0.16 μM) to alkaline pH oscillation in the M_1 subpopulation. Lysotracker Red D99 mediated pH-dependent trafficking showed a lower signal from the calcium-treated M_0 phenotype after 7 days of incubation, representing the M_1 phenotype. The higher pH is due to the lack of an active V-ATPase pump and increased proton consumption for superoxide dismutation. In contrast, M_2 lysosomes after calcium supplementation acidify rapidly with increased kinetics in phagosome maturation and hydrolysis, which is evident by LAMP-2 staining [33].

Several studies have reported that M_1 macrophages exhibit delayed maturation kinetics with late endosome fusion [34,35]. Due to delayed phagolysosome maturation, we obtained a lower signal from the ER tracker Red and Rab11 in calcium-supplemented M_0 macrophages, confirming its M_1 population. M_2 macrophage, after calcium supplementation, undergoes a rapid transition from early endosome to the phagolysosome maturation pool, which was evident in the M_2 group. This is the first study to simultaneously correlate MHC class II maturation and phagolysosome kinetics with the plasticity of differentiated macrophages following calcium supplementation.

It has been already established that differential cellular stimuli are responsible for maintaining cellular plasticity and differentiation of THP-1 cells [36]. Monocytes exhibit lower calcium uptake at day 1, whereas M0 and M2 macrophage shows higher calcium content with increased cytoplasmic volume. Here we have observed that M0 and M2 macrophage are maintaining their cellular plasticity up to 7 days in presence of calcium, without any growth factor. Increased intracellular calcium may leads to higher maturation of intracellular organelle. Sustained mobilization of intracellular calcium is responsible for activating cellular signalling pathway and induced cytokine release, leads to THP-1 maturation [37]. It has been already established that calcium ionophore treatment exhibit dendritic cell morphology, independent of prior differentiation state [38]. In our study we have observed that it not only helps to differentiate but also responsible for maintains its stable phenotypes.

CD-14 has been widely described as a monocyte/macrophage surface marker, which is present in all immune cell lineages [19]. It actively recognizes and activates the immune response in the presence of a foreign body [39]. The expression of CD-14 showed a progressive reduction in the presence of calcium ions in THP-1 monocytes and THP-1 M_0 macrophages in the presence of calcium ions. THP-1 M_2 macrophages showed an initial increase followed by a decrease in expression after calcium supplementation. THP-1 M_0 and THP-1 M_2 macrophages without calcium ions show elevated expression of CD14, as cells tend to detach and lose their differentiated morphology [40]. CD-36 and CD-68 are scavenger receptors expressed on the surface of THP-1 M_1 macrophages to promote phagocytosis [41]. CD-36 and CD-68 indicate the activation of pro-inflammatory macrophages and macrophage-specific promoters

[42]. THP-1 monocytes showed no significant expression of either CD-36 or CD-68; however, in the presence of calcium ions, they expressed CD-36 and CD-68. This was probably due to the induction of monocyte differentiation into macrophages or the presence of an M_0/M_1 mixed population [43]. The expression of the CD-36 surface marker increased in THP-1 M_0 macrophages, but no significant change was observed between days 3 and 7. CD-68 showed an initial elevation and then decreased expression in THP-1 M_0 macrophages. This indicates a decline in the phagocytosis of calcium ions by THP-1 M_0 macrophages [43]. The expression of IL-4 and IL-10 was increased in both THP-1 monocytes and THP-1 M_0 macrophages in the presence of calcium ions. M_0 macrophages exhibited a consistent increase in IL-4 and Il-10 levels due to the transition and M1/M2 population ratio changes. The THP-1 M_2 macrophages showed steady IL-4 and IL-10 levels, confirming a stable M_2 phenotype. The increase in IL-4 expression in THP-1 cells indicated the presence of calcium ions, which promoted their M_2-like morphology [44]. The increased release of nitric oxide and antioxidants in THP-1 cells may support IL-4 expression [45]. The increase in the expression of IL-10 was related to the expression of CD-36. CD-36 with p38 mitogen-activated protein kinase induces the expression of IL-10 surface markers. Therefore, THP-1 cells incubated with calcium ions showed an increase in the level of IL-10 surface marker.

To differentiate the M_0 macrophages from M_2 macrophages, the Immune complexes of IL-4 and IL-10 were used. IL-4 activates signal transducer and activator of transcription (STAT)-6, while IL-10 activates STAT1, STAT3, and STAT5 signalling pathways. Upon withdrawal of these chemicals or cytokines from differentiated THP-1 cells, they tend to detach and lose their differentiated morphology [40]. As observed in this study, THP-1 monocytes in the presence of calcium ions shifted from a single floating cell morphology to an attached, grouped morphology. This might be due to the homeostasis of calcium ions via calcium-sensing receptors present in THP-1 cells. In response, CaSR produces pro-inflammatory cytokines (nitric oxide and antioxidants) along with the expression of scavenging receptors on the cell surface (CD-36 and CD-68) [46]. Treatment of THP-1 monocytes with PMA activates the downstream process CaSR via the influx of calcium ions [47]. This indicates that the influx of extracellular calcium ions initiated the same targets as PMA.

5. Conclusion

The controlled temporal delivery of extracellular calcium ions could control the plasticity and polarization of human monocytes. Our study demonstrated the ability of calcium-mediated long-term programmed immune induction of specific macrophage subtypes. In addition, we identified an intermediate relationship between MHC class II maturation and cellular plasticity under various conditions. The intermediate relationship between reactive oxygen species, oxidative defence mechanisms, and cellular plasticity is inconclusive. We tried to find an answer regarding the possible use of calcium to control cellular plasticity and state of differentiation. It will help to design different future calcium-releasing biomaterials by combining calcium to control cellular plasticity and signalling in tissue regeneration and engineering.

Author agreement

SR performed a benchwork and polarized THP-1 cells, co-cultured them, generated a stable phenotype and designed the experimental outline. AS helped with confocal microscopy. SG conceived of the idea and supervised the study. SG prepared the final manuscript.

Data availability

All raw data generated or analysed during the current study are available from the corresponding author upon reasonable request by maintaining privacy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.12.054.

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