Journal of Cell Identity
volume X, number 1
spring 2020

Normal human monocytic cell line CRL9855 can be transformed into macrophages with very low concentrations of phorbol 12-myristate 13-acetate

Journal:	*Journal of Cell Identity*
Manuscript ID	CELLID-2021-0002.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	31-Aug-2021
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Keywords:	monocytes, PMA, differentiation protocol, macrophages, CRL-9855, SC cell line

We thank the reviewers for the time spent evaluating our manuscript, as well as for their constructive comments. Please find below the modifications made point by point. They were also introduced in the manuscript with TrackChanges.

Reviewer 1:

Comments to the Author:

The article is well structured, with a clearly stated line of inquiry and with a few modifications, I recommend it for publication:

- page 3, line 9, "it can be used for, for example, for exporation..." avoid the alliteration

The sentence was modified to: “It can be used for exploration…”

- line 43, replace "phagocyting" with "phagocytosis"

- replaced as indicated

-page 4, line 23 "PMA induction of this cell line" replace with "PMA induced macrophage differentiation of this cell line"

- replaced as indicated: “a limited number of studies have investigated the optimum conditions needed for PMA induced macrophage differentiation of this cell line”

-line 26, "some articles report an nM concentration", replace with "some articles report a nM concentration"

- the correction has been made

And last, but not least, in material and method chapter, the expression is a bit ambigous about the concentrations used. were they calculated in tubes, before adding them into the plate's wells, or was it the calculated concentration in the well. (for example, if you'd have a 200 ng/mL solution and you'd add 20 µL into the well of a 96 well plate and add 180 µL of growth medium, the final concentration would ben 20 ng/mL). Make it a bit more clear which were the working solutions concentrations and which were the concentrations in the plate with the cells.

The reviewer raised a very good point. We prepared in tubes 2x solutions, which were further added to wells already seeded with the desired number of cells (PMA solution/well medium -1:1 vol/vol). The final concentrations in the wells (working concentrations) are the ones indicated in the manuscript.

To clarify this point, we modified this section of Material and Method as follows.

“2x working solutions were prepared by successive dilutions in 1.5 mL tubes, using complete cell medium. Each PMA solution was further diluted in-well, 1:1 with an equal volume of cell medium containing the desired number of cells. The final in-well working concentrations of PMA were 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 10 ng/mL and 5 ng/mL”

Reviewer 2:

Pg1, line 44, 52: Usually when referring to a cell line, the cell line designation name is used, I recommend replacing the reference number CRL-9855 with its name which is SC. The product code (ATCC-CRL-9855) can appear only in the Materials and methods section. If the reference number is maintained at least should be written in the same way, different variants are found in the manuscript text: e.g., CRL-9855, 9855-CRL.

We thank the reviewer for the suggestion - we changed the reference number with the name of the cell line, as recommended. The reference number was only mentioned in the "Materials and method" section.

Pg3, line 9: To avoid repetition of "for" the authors can rephrase the sentence in this way: For instance/In particular, it can be used for exploration.....

The sentence was modified to: "It can be used for exploration…"

Pg4, line 51: the abbreviation IMDM should be explained Symbol missing: Pg5, line 5: µL (microliter) instead of uL; Pg4, line 54: 30°C instead of 30C Space missing: e.g. 10%fetal, 100µM, 16µM (Pg4, line 52); 12hrs (Pg5, line 12); 200nM (Pg5, line 35), etc.

We performed the recommended changes.

Pg5, line 21: both "min" and "mins" are used in the same sentence. The authors should keep only one abbreviation.

We used "mins" throughout the article

Pg5, line 45: I suggest "SC human monocytes" instead of "9855 human monocytes"

Changed to "Phase-contrast images of PMA treated SC human monocytes at 24 and 48 hrs."

Pg6, lines 19,21,54; pg7, line7, 28: "hrs" instead of "hours"

We replaced "hrs" instead of "hours" throughout the manuscript

In fig.1 should be indicated which cells are after 24 hrs and which are after 48 hrs. I suggest also adding images of untread cells as control.

In fig.2 scale bar is missing for the right panel. Also, in the figure caption, should be indicated what the white arrows are pointing to.

In fig.3 is not clear which concentration corresponds to which line. What significance has the right panel in black color?

We reedited all three figures according to suggestions. Arrowheads of figure 2 point at dead cells/cell fragments, we added the information in figure caption.

To increase the visibility/impact of the paper I recommend insisting more on the advantages and utilization of the SC macrophages in different studies /applications.

We thank the reviewer for the suggestion. Although monocytes/macrophages are frequently used in preclinical studies, usually as an inflammation model, or to test the pro-inflammatory potential of various drugs, most research groups prefer to use THP-1 cell line. Because unlike THP-1, SC cell line is from a normal background, it would be more suited for toxicity testing, for example. We added the following paragraph in Discussion Section

”We believe that for assessment of biocompatibility (e.g. toxicity, inflammation studies) a normal cell line would be a preferable choice over a tumor cell line. Therefore, we focused on a normal monocyte line, SC monocytes. This particular cell line is not well referenced in the literature – a search on PubMed using ”CRL-9855”) retrieve only 3 results and we found an additional one using ”SC cell line”. A search for ”SC macrophages” or ”SC monocytes” was not very informative, as the name of the cell line (SC) was frequently omitted from the search. Of the four articles, only one reported the use of PMA at 20 ng/mL (Ribeiro et al., 2014).”

Normal human monocytic cell line CRL9855 can be transformed into macrophages with very low concentrations of phorbol 12-myristate 13-acetate

Running title: Phenotype change of 9855 monocytes with low concentrations of PMA

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Abstract

Monocytes are leukocytes that can differentiate into tissue macrophages and act in immune surveillance and tissue reconstruction. They are frequently used in vitro to study inflammatory response, lymphocyte activation, and phagocytosis. A widely used model is the in vitro differentiation of various monocytic cell lines including THP-1 and U937 using phorbol 12- myristate 13-acetate (PMA). These cell lines have various limitations because of their malignant background. Data available on utilizing normal human monocytes cell lines are scarce. Our study aims to identify the optimum concentration of PMA needed to maximize the number of adherent macrophages using the normal monocytic cell line ~~CRL-9855~~SC (ATCC CRL-9855). Using impedance readings and time-lapse microscopy, we determined that the optimum PMA concentration is 25ng/mL for 48 ~~hours~~hrs. Using these optimized conditions, we were able to induce the formation of adherent and proliferating macrophages, which can be further used for morphology and functional studies.

Keywords: CRL-9855, SC cell line, monocytes, PMA, differentiation protocol, macrophages.

List of symbols and abbreviations:

CMP - common myeloid progenitor
GM-CSF - granulocyte-macrophage colony-stimulating factor
G-CSF - granulocyte colony-stimulating factor
LPS – lipopolysaccharide
PMA - phorbol 12-myristate 13-acetate

Introduction

Monocytes are part of the reticuloendothelial system, along with tissue-resident macrophages. Like most cells of the immune system (except for lymphocytes), monocytes originate from the multipotential common myeloid (CMP) progenitor stem cell from the bone marrow, under the influence of cytokines: GM-CSF (Granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating), and IL-3. GM-CSF is produced by a large category of cells, like endothelial cells, T cells, macrophages, mast cells, and fibroblasts (Ross and Pawlina, 2020). Following differentiation into adult monocytes, they enter the bloodstream and migrate into tissues under the control of local growth factors, pro-inflammatory cytokines, and microbial products. Now, they can differentiate into local macrophages, such as osteoclasts, alveolar macrophages, or Kupffer cells (Ross and Pawlina, 2020). Monocytes are involved in various immune processes: they play a very important role during bacterial, protozoan, and viral infections, in cancer, atherosclerosis, and autoimmune diseases. For example, they can provide effective protection against infection with gram-negative bacteria, by interaction with lipopolysaccharide (LPS), a macromolecule found in the bacterial wall. Peripheral monocytes migrate to lymph nodes where they become antigen-presenting cells for CD4+ and CD8+ T cells, which can initiate adaptive immunity against pathogens, mainly via cytokine production (Bosshart and Heinzelmann, 2016).

Due to their important role in immune responses, monocytes are frequently used as an *in vitro* model to study inflammatory response, lymphocyte activation, and phagocytosis. There are several known cell lines, frequently reported in original articles, such as THP-1 or U937 cell lines. The THP-1 cell line is a human monocytic leukemia cell line, obtained

from a patient who suffered from acute monocytic leukemia. THP-1 cells can start to adhere to culture plates and can be cultured in vitro to passage 25 and still keep their sensitivity and activity (Bosshart and Heinzelmann, 2016). U937 is a pro-monocytic human myeloid leukemia cell line, isolated from a patient who suffered from histiocytic lymphoma. It can be used ~~for, for example,~~ for exploration of the mechanisms behind the attachment of monocytes to the endothelium. Unlike THP-1, U937 can be used for a higher number of passages. It is important to know that these cell lines have some limitations because of their malignant background (Chanput, Peters and Wichers, 2015).

One main application of monocytic cell lines is their conversion into macrophages. The most common protocols used involve phorbol 12-myristate 13-acetate (PMA) (Daigneault *et al.*, 2010). It is very important to mention that, at first, PMA induces inhibition of cell growth. More exactly, it can inhibit the cell cycle at G1-phase via a complex mechanism which includes the modulation of the expression of some cell cycle regulators, initiated by the cellular generation of reactive oxygen species (Traore *et al.*, 2005).

The mechanism through which PMA can act is through the activation of protein kinase C. An advantage of using PMA is that it is not dependent on receptors on the cell membrane and it is less time-critical, as its effects are sustained for at least 30 minutes. As far as cell morphology goes, it has been proven that the number of some organelles is different and the cytoplasm can expand (this modifies the ratio between cytoplasm and nucleus, favoring the cytoplasm). For example, cells treated with PMA followed by a period of 5 days resting in culture without PMA increased the number of lysosomes and mitochondria. Also, these cells are more resistant to apoptosis and the capacity of ~~phagocyting~~ phagocytosis is higher, especially for latex beads (Daigneault *et al.*, 2010). It has been shown that PMA differentiation induces the expression of genes related to PI-3K signaling pathway and phagosomal pathway (Zeng *et al.*, 2015). However, it has been reported that differences may exist between U937 and THP-1 in terms of phagocytic activity (Mendoza-Coronel and Castañón-Arreola, 2016), while THP-1 was similar in this respect to human circulating monocytes (Madhvi *et al.*, 2019). These differences could be explained by numerous chromosomal aberrations (Adati *et al.*, 2009), and mutations detected in THP-1 cells (https://maayanlab.cloud/Harmonizome/gene_set/THP-

1/COSMIC+Cell+Line+Gene+Mutation+Profiles), as well as U937 cells (https://maayanlab.cloud/Harmonizome/gene_set/U937/CCLE+Cell+Line+Gene+Mutation+Profiles).

From this perspective, the optimum model would be primary culture of macrophages derived from patients' circulating monocytes. However, as patient sampling is not always available, a cell line from a normal genetic background could provide the best *in vitro* model. Such model is provided by ~~CRL-9855~~SC cell line, obtained from immature monocytes, with no malignant characteristics (US Patent 5,447,861).

The data available regarding the PMA-induced differentiation protocol for this cell line is scarce. This cell line provides the benefit of normal background and may offer a different landscape during activation of inflammatory mechanisms compared to malignant cells.

In literature, a limited number of studies have investigated the optimum conditions needed for ~~PMA induction of this cell line~~ PMA induced macrophage differentiation of this cell line (Wawrzynkiewicz *et al.*, 2020)(Samie *et al.*, 2016). Additionally, there is no consensus on the optimum starting concentration for this PMA differentiation protocol. Some articles report ~~an~~ a nM concentration, whereas others, ng/mL. Either way, the working concentration ranges between 10-200 either nM or ng/mL. (Daigneault *et al.*, 2010)(Starr *et al.*, 2018)(O'Mahony *et al.*, 1998)(Park *et al.*, 2007). Also, in these studies a different number of cells is used, varying between $5*10^4$ and $2*10^6$ cells/µL.

Our study aims at investigating the optimum concentration of PMA to achieve the maximum number of adherent macrophages using the normal monocytic cell line ~~CRL-9855~~SC. Given the changes induced by PMA treatment, we followed a scale-down approach, starting from the highest concentration reported in the literature (200 ng/mL), to obtain the best result with minimum changes in cell proliferation status and offer a starting point for further research.

Material and method

Cell culture and cell treatments. Normal human monocytes (~~9855-CRL, ATCC~~ATCC-CRL-9855~~)~~ were routinely maintained according to manufacturer's instructions Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum, 1% antibiotic, hypoxanthine (100µM) and thymidine (16 µM) (HT supplement 100x) and 55

µM beta-mercaptoethanol) in a 5% CO2 incubator and 37°C. Cells were treated with PMA (Sigma Aldrich P1585), reconstituted in DMSO to a concentration of 1 mg/mL (1st stock). Following the first utilization of one stock vial, a second stock solution was prepared (0.1 mg/mL) which was aliquoted in 10 µL~~uL~~ aliquots for single (or maximum 2) use. ~~Working solutions were prepared prior to use, by dilution in complete cell medium (200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 10 ng/mL and 5 ng/mL)~~ 2x working solutions were prepared by successive dilutions in 1.5 mL tubes, using complete cell medium. Each PMA solution was further diluted in-well, 1:1 with an equal volume of cell medium containing the desired number of cells. The final in-well working concentrations of PMA were 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 10 ng/mL and 5 ng/mL. 50 000 cells were plated in 24 wells plates and incubated for up to 72 hrs in decreasing concentrations of PMA. Images were captured every 24 hrs using a phase-contrast Evos microscope.

Real-time impedance readings: Cell adhesion and cell proliferation were assessed using RTCA DP platform (Agilent Technologies). They were trypsinized and seeded in E-16 plates at a density of 50000 cells/well, with or without PMA at indicated concentrations. Readings were collected every 5 mins for 24 h, followed by readings at 15 mins up to 92 hrs.

Videomicroscopy: 4 chambers 35mm dish (Ibdi, µ-Dish 35 mm Quad - 80416) were seeded with 10000 cells/well and recorded every 15 mins for 48 hrs in a Biostation Incubator (Nikon). Postdata analysis was performed using Biostation IM software (Nikon).

Results

1. Normal human monocytes require 48 hrs to adhere, at low concentrations of PMA

Frequently, phenotype change is induced with either 200 nM or 200 ng/mL, therefore 200 ng/mL was the starting concentration of our study which corresponds to 324,24 nM (Table 1). 24 hrs were insufficient for cell adherence, although adhered cells could be occasionally observed. At 48 hrs adherent cells were observed for concentrations as low as 25 ng/mL (fig1).

Fig.1 Phase-contrast images of PMA treated ~~9855 human monocytes~~SC human monocytes at 24 and 48 hrs. Arrows indicate adhered cells. 20x magnification. Scale bar 100 µm.

Table 1. Correlation between various concentrations of PMA used in experimental approaches

PMA (ng/mL)	PMA (nM)
200	324.24
100	162.12
50	81
25	40.5

Next, we were interested in defining the timeline of monocyte adherence, hence we performed a time-lapse recording of the modifications induced by various concentrations of PMA on monocyte phenotype (fig.2). We observed that at high concentrations (200,100 ng/mL) mitosis does not occur during the first 24-27 ~~hours~~hrs, whereas for the low concentrations, cells begin to divide after the first 6-7 ~~hours~~hrs of treatment (fig.2 left panel), albeit many cell deaths are also observed during the first 24 hrs (fig.2 right panel).

Fig.2. Selected frames of time-lapse recordings of monocytes treated with 25 ng/mL PMA. Left panel includes 3 successive time-frames (steps 46-48, at 15 mins apart). Right panel is captured after 15 hrs of treatment with the same concentration. Arrowheads point at dead cells/cell fragments. Phase contrast, 20x. Scale bar 10 µm.

Finally, we were interested in quantifying the number of cells that adhered and can be further used for functional experiments. We chose to use the xCelligence real-time platform, which can record and quantify in real-time the contacts the cells make with the plate bottom (fig3).

Fig.3. Real-time impedance readings of PMA treated ~~9855 human monocytes~~SC human monocytes. Each concentration was tested in triplicate, using 50000 cells/well. Graph lines represent impedance readings at every 15 mins ± S.D for each reading.

As expected, untreated cells recorded a very low index, whereas PMA treated cells displayed a reversed dose-dependent response. Of note, no significant change was observed between tested concentrations during the first 15 ~~hours~~hrs. The cells treated with 200 ng/mL entered a plateau around 24 hrs which lasted for at least another 24 hrs, then resumed proliferation at a slow rate. The appearance of this proliferation plateau decreased with decreased concentration. The lowest concentration allowed the proliferation of adhered cells up to 90 ~~hours~~hrs.

Discussion

Phenotype change of circulating monocytes in macrophages under PMA treatment is widely used in *in vitro* studies of inflammation and immune responses. However, there are two major issues regarding this protocol: the variety in PMA concentration and incubation time. More consistency in differentiation protocol is required (Park *et al.*, 2007). Also, cells with malignant background are frequently used, whereas normal human cell lines are less frequently reported or studied. "We believe that for assessment of biocompatibility (e.g. toxicity, inflammation studies) a normal cell line would be a preferable choice over a tumor cell line. Therefore, we focused on a normal monocyte line, SC monocytes. This particular cell line is not well referenced in the literature – a search on PubMed using "CRL-9855") retrieve only 3 results and we found an additional one using "SC cell line". A search for "SC macrophages" or "SC monocytes" was not very informative, as the name of the cell line (SC) was frequently omitted from the search. Of the four articles, only one reported the use of PMA at 20 ng/mL (Ribeiro *et al.*, 2014).

Our study demonstrated that normal human monocytes can become adherent with low PMA concentrations, and this phenotype change does not affect cell proliferation. Scaling down PMA concentrations still induced cell adherence in our experimental setup, as soon as 24 ~~hours~~hrs. However, for functional studies, especially cytokine assessment, a minimum number of cells is necessary to synthesize a minimum amount of protein detectable in various protein assays. In order to quantify cell number, we used impedance reading assay, which converts cell number into a measurable cell index. Low

concentrations of PMA were correlated with the highest cellular index after 3 days of cell culture. As reported in the literature, PMA treatment induces a stop in G1 (Spano, Barni and Sciola, 2013), which is translated as a plateau in our impedance recordings. As observed, higher PMA concentrations induce a longer plateau, whereas at lower concentrations, the plateau phase is reduced and cell proliferation is resumed sooner. The rising of the index indicates that proliferating cells are still attached to the bottom of the plate. Videomicroscopy confirmed that the inhibition of cell cycle is shorter with lower concentrations of PMA and cells are resuming cell divisions faster, although failure of cell division and subsequent cell death was observed during the first 24 hrs. After 48 hrs, an adherent population of dividing cells is obtained, for further studies.

In conclusion, we demonstrated that normal human monocytes ~~CRL-9855~~SC treatment with 25 ng/mL of PMA is a better option for phenotype change into adherent macrophages. As future line of investigation, the evaluation of the phenotype of the macrophages produced by our protocol could be a next step.

Funding: This work was supported by a grant of the Ministry of Research, Innovation and Digitization, CNCS/CCCDI – UEFISCDI,, in the framework of project PN-III-P1-1.2-PCCDI-2017-0062 (contract no. 58) and PNIII.P2-2.1-PED-2019-3141 (contract no. 382).
Conflict of interest: The authors declare no conflict of interest.

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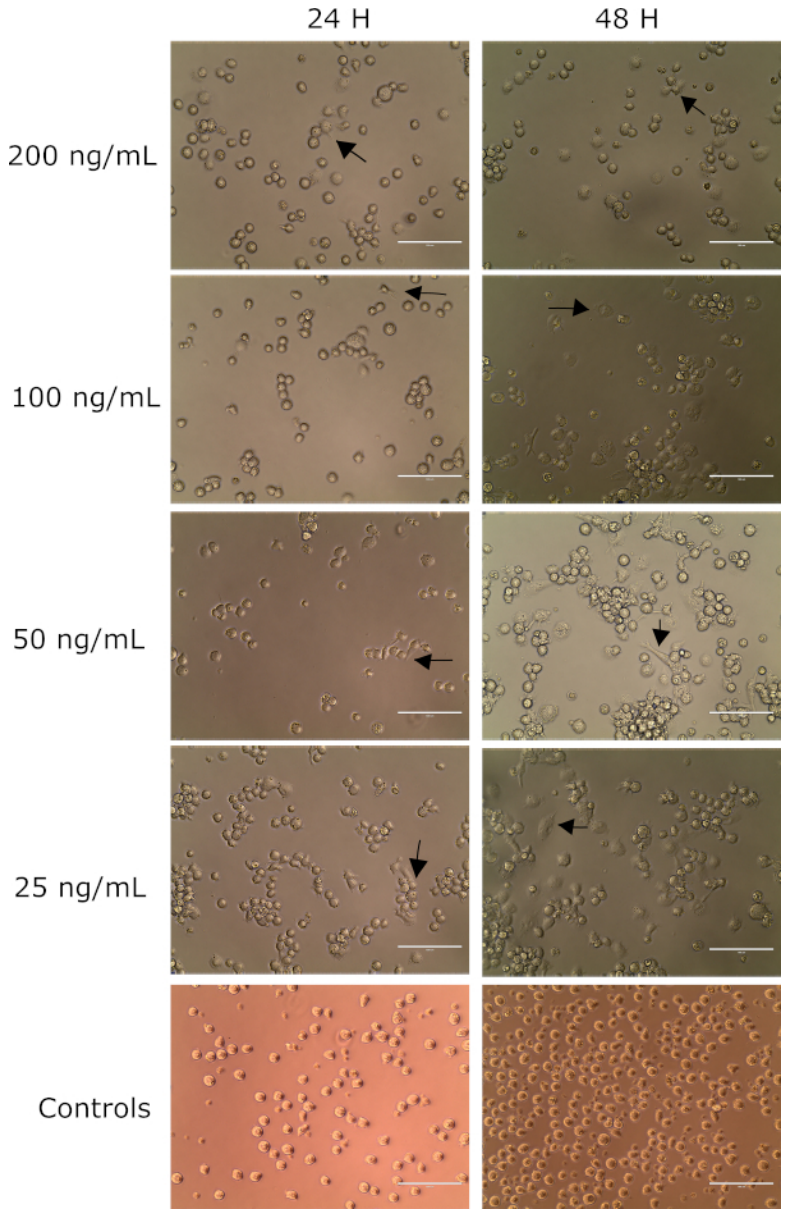

Phase-contrast images of PMA-treated SC human monocytes at 24 and 48 hrs. Arrows indicate adhered cells. 20x magnification. Scale bar 100 µm.

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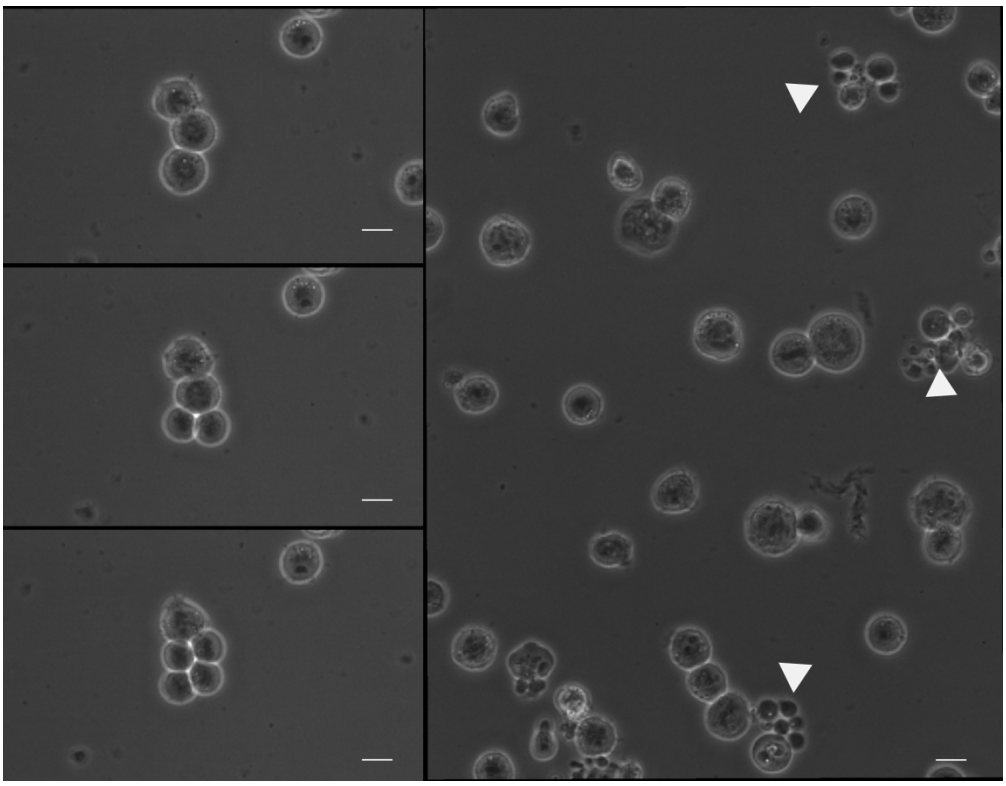

Selected frames of time-lapse recordings of monocytes treated with 25 ng/mL PMA. Left panel includes 3 successive time-frames (steps 46-48, at 15 mins apart). Right panel is captured after 15 hrs of treatment with the same concentration. Arrowheads point at dead cells/cell fragments. Phase contrast, 20x. Scale bar 10 μm.

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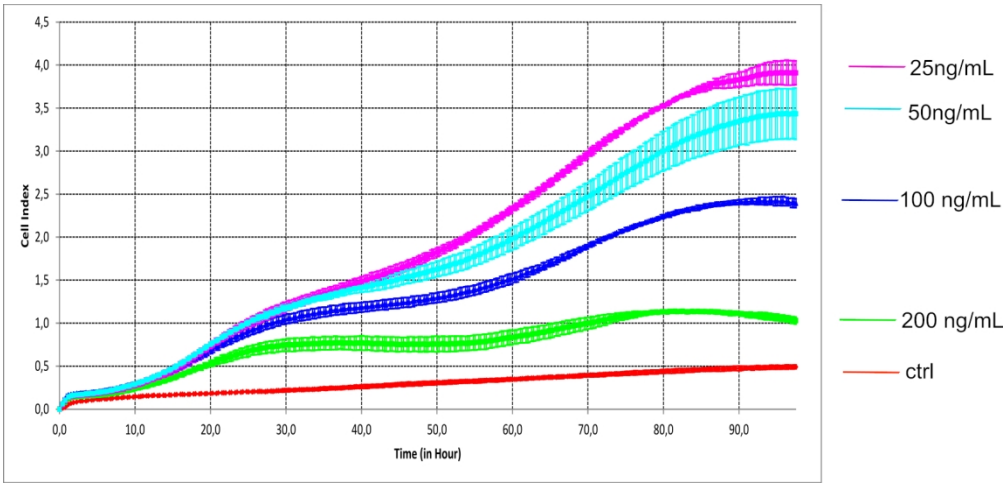

Real-time impedance readings of PMA treated SC human monocytes. Each concentration was tested in triplicate, using 50000 cells/well. Graph lines represent impedance readings at every 15 mins ± S.D for each reading.

457x219mm (96 x 96 DPI)