General comments

In this study Simakou et al, set out to examine the effects of vitamin D3 administration, vibrational stimulation and their combined effect on THP-1 cells with a focus on their differentiation potential into macrophages. A series of RT-qPCRs were conducted to measure the various gene expression changes associated in response to stimuli (vitamin D3 vs 1000Hz or both) and cell condition (suspended vs adherent). The data presented shows novelty and proficiency that should make this manuscript eligible for publication in PeerJ.

The data is efficiently summarised and presented well in both graph and table format which allows clear comparison and interpretation of the data. The authors show novel expression patterns such as PKD2 upregulation with vitamin D3 administration. With further testing in other cell types, this may serve as unique signature of vitamin D3 stimulation.
A comment on potential limitations of THP-1 immortalised cell line vs primary cell lines may be warranted in order to acknowledge the need for further research and confirm findings in the future. In order to enhance readability, the authors should significantly improve sentence structure and avoid grammatical errors. This will avoid breaking the flow of the text and hasten understanding of key points presented.

1) The methodology should be clearer. For example, over the 72-hour period, how was vitamin D3 administered? Is it given once in the medium and left for 3 days? Is it replenished after each day?

Did the authors look at *PIEZO2* in THP-1 cells? It may be informative to include the sibling of PIEZO1 for comparative purposes. RT-qPCR analysis of this mechanosensor may be included across vitamin D3 treated, 1000Hz and combined conditions.
Alternatively, the reason why it was not analysed should be presented e.g., future studies will do so; or based on previous data PIEZO2 is not highly expressed in THP1-cells (Reference).

The authors summarise the data elegantly using tables (Tables 1, 2 and 3); however, the main text makes little to no reference of this. Guiding readers to use these tables as comparisons are made may help with the flow of data presentation and discussion points.

Conclusion should be added to abstract. Currently the abstract abruptly ends on Results. A clear "**conclusion:**" with a sentence or two to summarise the main findings should be included.

The figure legend for Figure 4 should be revised. I believe this refers to adherent cell data yet suspension is written.
Figure legend for Table 2 should be revised. I believe this is comparison of **stimulated** adherent THP-1 cells vs unstimulated adherent cells

Improve English and sentence structure throughout manuscript e.g., repetitive use of the phrase “the vitamin D3” can simply be replaced with “vitamin D3” for example line 288 “The vitamin D3 downregulated..” instead “Vitamin D3 downregulated..” may be used.

Improve sentence structure in lines 609-610 “This study had limitation because of the technology” – e.g., This study was limited by the technology which…
line 73-74; improve sentence structure. Maybe break the sentence in two

2) Using only one vibrational parameter (1000Hz) may limit potential mechanosensory responses. For example, previous studies (Koizumi et al, 2014; Functional role for Piezo1 in stretch-evoked Ca^{2+}influx and ATP release in urothelial cell cultures. J Biol Chem 289 (23):16565-16575) have shown the presence of a stretch stimulated threshold that must be attained before mechanosensor mediated changes in cell responses (ATP efflux) are observed. Similarly, the current study may yet to uncover a potential vibrational range that alters gene expression differently than reported. Is there a spectrum of gene expression changes that occurs by changing the range of vibration (Hz, time, cyclical stimulation?). Is there a range in which macrophage differentiation is promoted? Furthermore, the nature of mechanical stimuli (vibration vs stretch vs hydrostatic pressure changes) may alter THP-1 cell`s gene expression. The 1000Hz is a standard range used in THP-1 cells thus it is understandable why the current report did not alter it. However, commenting on these issues may help the reader understand the dynamic range of cell responses that have yet to be elucidated. For the current scope of this report additional experiments are not essential (specially as the authors explained the limitation; lines 609-610), however acknowledgement or dismissal of such potential changes using previously published data or inference from data presented by the study should be presented to the reader.

3) It would be informative to compare basal expression levels of key genes in suspended and adherent conditions. For example, do adherent cells express comparably higher levels of PIEZO1 than unstimulated suspended cells? Are the levels of CD14 and CD36 higher in adherent vs suspended cells?

4) Perhaps beyond the scope of the current study, but analysis of protein expression levels of key macrophage differentiation markers (CD14, CD36 etc.) through western blot may strengthen the claims made. The authors could state the need to confirm their key findings at the protein level to corroborate their RT-qPCR data.

Gene expression during THP-1 differentiation is influenced by vitamin D3 and not vibrational mechanostimulation (#58110)

First submission

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- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- Impact and novelty not assessed. Negative/inconclusive results accepted. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- All underlying data have been provided; they are robust, statistically sound, & controlled.
- Speculation is welcome, but should be identified as such.
- Conclusions are well stated, linked to original research question & limited to supporting results.

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Tip	*Example*
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
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Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Gene expression during THP-1 differentiation is influenced by vitamin D3 and not vibrational mechanostimulation

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Background: In tissue injury or infection, monocytes migrate into the affected tissues from circulation and differentiate into macrophages which are subsequently involved in the inflammatory responses. Macrophage differentiation and activation have been studied in response to multiple chemokines and cytokines. However, the physical properties of tissues such as stiffness or pressure, also influence the macrophage differentiation, activation, cytokine production, and phagocytic activity. **Methods:** In this study, the macrophage differentiation from THP-1 monocytes was assessed upon the stimulation with 1,25-dihydroxyvitamin D3 and 1000Hz vibration, using qPCR for quantification of transcript expression. The vitamin D binds the vitamin D receptor (VDR) and subsequently modulates the expression of a variety of genes in monocytes. The effects of the 1000Hz vibrational stimulation, and the combined treatment of vitamin D3 and 1000Hz vibrations were unknown. The differentiation of macrophages was assessed by looking at transcription of macrophage markers (e.g. *CD14*, *CD36*), transcription factors (e.g. *LEF-1*, *TCF7L2*), antigen presenting molecules (e.g. *HLA-DRA*), and mechanosensors (e.g. *PIEZO1* and *PKD2*). **Results:** The results showed that the vitamin D3 induced THP-1 macrophage differentiation, which was characterised by upregulation of *CD14* and *CD36*, downregulation of *HLA-DRA*, upregulation of the *PKD2* (*TRPP2*), and an inverse relationship between *TCF7L2* and *LEF-1*, which were upregulated and downregulated respectively. The downregulation of *HLA-DRA* in early differentiation induced by the vitamin D3 could also show predisposition for development of M2 polarised phenotypes. The 1000Hz vibrations were sensed from the cells which upregulated *PIEZO1* and *TCF3*, but they did not induce expression of genes that would indicate macrophage differentiation. The mRNA transcription profile in the cells stimulated with the combined treatment was comparable to that of the cells stimulated by the vitamin only. The 1000Hz vibrations slightly weakened the effect of the vitamin for the regulation of *CD36* and *HLA-DMB* in the suspension cells, but without causing changes in the regulation patterns. The only

Abbreviation list may be useful

exception was the upregulation of *TCF3* in the suspension cells, which was influenced by the vibrations. In the adherent cells, the vitamin D3 cancelled the upregulating effect of the 1000Hz vibrations and downregulated *TCF3*. The vitamin also cancelled the upregulation of *PIEZO1* gene by the 1000Hz vibrations in the combined treatment.

For ease of read
Add conclusion in abstract

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Abstract

Background: In tissue injury or infection, monocytes migrate into the affected tissues from circulation and differentiate into macrophages which are subsequently involved in the inflammatory responses. Macrophage differentiation and activation have been studied in response to multiple chemokines and cytokines. However, the physical properties of tissues such as stiffness or pressure, also influence the macrophage differentiation, activation, cytokine production, and phagocytic activity.
Methods: In this study, the macrophage differentiation from THP-1 monocytes was assessed upon the stimulation with 1,25-dihydroxyvitamin D3 and 1000Hz vibration, using qPCR for quantification of transcript expression. The vitamin D binds the vitamin D receptor (VDR) and subsequently modulates the expression of a variety of genes in monocytes. The effects of the 1000Hz vibrational stimulation, and the combined treatment of vitamin D3 and 1000Hz vibrations were unknown. The differentiation of macrophages was assessed by looking at transcription of macrophage markers (e.g. *CD14*, *CD36*), transcription factors (e.g. *LEF-1*, *TCF7L2*), antigen presenting molecules (e.g. *HLA-DRA*), and mechanosensors (e.g. *PIEZO1* and *PKD2*).
Results: The results showed that the vitamin D3 induced THP-1 macrophage differentiation, which was characterised by upregulation of *CD14* and *CD36*, downregulation of *HLA-DRA*, upregulation of the *PKD2* (*TRPP2*), and an inverse relationship between *TCF7L2* and *LEF-1*, which were upregulated and downregulated respectively. The downregulation of *HLA-DRA* in early differentiation induced by the vitamin D3 could also show predisposition for development of M2 polarised phenotypes. The 1000Hz vibrations were sensed from the cells which upregulated *PIEZO1* and *TCF3*, but they did not induce expression of genes that would indicate macrophage

differentiation. The mRNA transcription profile in the cells stimulated with the combined treatment was comparable to that of the cells stimulated by the vitamin only. The 1000Hz vibrations slightly weakened the effect of the vitamin for the regulation of *CD36* and *HLA-DMB* in the suspension cells, but without causing changes in the regulation patterns. The only exception was the upregulation of *TCF3* in the suspension cells, which was influenced by the vibrations. In the adherent cells, the vitamin D3 cancelled the upregulating effect of the 1000Hz vibrations and downregulated *TCF3*. The vitamin also cancelled the upregulation of *PIEZO1* gene by the 1000Hz vibrations in the combined treatment.

Introduction

Macrophages play important roles in health and disease through phagocytosis of pathogenic microorganisms, by releasing inflammatory mediators, by inducing and maintaining inflammation, and by removing apoptotic cells and repairing tissues (Gordon, 2007; Mosser and Edwards, 2008). Macrophages already present in specific tissue are called tissue-resident macrophages (Lavin *et al.*, 2015). Tissues-resident macrophages are derived from the yolk sac at the embryonic stage, are replicated in tissues to maintain cell number, and have different morphology and function depending on the tissue where they reside (Lavin *et al.*, 2015). Such macrophages include the Kupffer cells in the liver and macrophage-like microglia in the nervous system. However, in the case of tissue injury or infection, monocytes derived from bone marrow circulating in peripheral blood migrate to the affected tissues where they differentiate into macrophages, and are subsequently involved in the inflammatory response (Shi and Pamer, 2011).

THP-1 cells are human immortalised monocytes derived from acute monocytic leukaemia and have been extensively used to study macrophage differentiation, functions, signalling pathways, and nutrient and drug transport (Chanput, Mes and Wichers, 2014; Bosshart and Heinzelmann, 2016). In this study we investigate the THP-1 responses towards stimulation with vitamin D3 (1,25-dihydroxyvitamin D3), 1000Hz nanovibrations or both, in order to study the expression of genes that could indicate differentiation or mechanosensitivity changes in these cells. In the following text, the combined treatment refers to the combination of 50 nM vitamin D3 and 1000Hz vibrations. The cell responses were investigated in both adherent and suspension THP-1 monocytes upon each stimulation, in order to avoid any confusion in results due to potential differences between the cell types within the same population. improve sentence structure

Vitamin D has been shown to promote monocyte differentiation into macrophages and targets multiple genes (Nurminen, Seuter and Carlberg, 2019). The active form of vitamin D, 1,25-dihydroxyvitamin D3, is a lipophilic molecule which easily passes through biological membranes and binds with high-affinity to the receptor and transcription factor vitamin D receptor (VDR), which is primarily located in the nucleus

(Haussler *et al.*, 2013). The activation of vitamin D target genes is explained by the chromatin model (Nurminen, Seuter and Carlberg, 2019). The ligand-activated VDR molecules bind to a wide variety of enhancer regions that carry suitable binding motifs and are located within accessible chromatin. With the help of pioneer factors, such as PU.1, CEBPA, and GABPA, VDR increases the accessibility of chromatin at and around these enhancer regions (Seuter, Neme and Carlberg, 2017, 2018; Nurminen *et al.*, 2019). In THP-1 cells, 1,25-dihydroxyvitamin D3 stimulation significantly affects the binding strength of transcription factor CTCF to topologically associating domain (TAD) anchors, which results in about 600 TADs becoming sensitive of (to?) vitamin D (Neme, Seuter and Carlberg, 2016). Looping of activated DNA-bound VDR to a transcription start site (TSS) at these promoter regions results in increased chromatin accessibility as well as of H3K27ac and H3K4me3 marks (Seuter, Neme and Carlberg, 2016; Nurminen *et al.*, 2019). All these vitamin D-triggered changes in the local chromatin structure at enhancer and promoter regions finally lead to the activation of RNA polymerase II assembled on the respective TSSs and the start of mRNA synthesis. The vitamin may also affect gene expression by increasing the expression and the activity of transcription factors other than VDR, such as BCL6, NFE2, POU4F2, and ELF4 (Nurminen *et al.*, 2015).

The effects of vitamin D have been studied in the context of macrophage differentiation from monocytes, but they are also extended into the effector macrophage responses (Hewison, 2010) . In fact,(space) normal human macrophages are able to synthesize 1,25-dihydroxyvitamin D3 when stimulated with interferon gamma (IFNγ) (Phillip Koeffler *et al.*, 1985). The vitamin D is involved in the regulation of T cell and macrophage effector functions, primary (primarily?) via localized autocrine or paracrine synthesis of 1,25-dihydroxyvitamin D3 from its precursor 25-hydroxyvitamin D_3 (Hewison, 2010). In addition, vitamin D deficiency is prevalent in multiple autoimmune diseases, such as multiple sclerosis, type 1 diabetes, systemic lupus erythematosus, and alopecia areata, and it is highly associated with the risk of autoimmunity (Yang *et al.*, 2013; Lin, Meng and Song, 2019). Vitamin D has been implicated in prevention and protection from autoimmune diseases by immunomodulation of macrophage, dendritic cell, and T cell responses (Hewison, 2010; Yang *et al.*, 2013).

In the recent years, interest has been given to the mechanobiology of macrophages, which like other immune cells have evolved mechanisms to perceive and respond to the mechanical forces around them (Kim *et al.*, 2019). The cellular functions of tissue-resident macrophages and monocyte-derived macrophages are affected by the tissue-specific microenvironment, which can create many types of mechanical stress on cells (McWhorter, Davis and Liu, 2015; Mennens, van den Dries and Cambi, 2017). Stiffness and topography, which are mechanical properties of the extracellular matrix, regulate the differentiation, proliferation, and function of macrophages such as phagocytosis (Patel *et al.*, 2012). In monocytes, the PIEZO1 mechanotransduction in response to

cyclical hydrostatic pressure, results in HIF1α stabilization and secretion of molecules, such as endothelin-1 (EDN1), and neutrophil chemoattractant CXCL2 (Solis *et al.*, 2019). In addition, macrophages in tissues are exposed to alterations of pressure which affect the secretion of cytokines such as IL-6, TNF-α and IL-1β, (Ferrier *et al.*, 2000; Mevoy *et al.*, 2002). Other mechanical forces that these cells experience originate from dynamic mechanical loading, such as continuous and cyclic stretch and compression (McWhorter, Davis and Liu, 2015; Mennens, van den Dries and Cambi, 2017).
Just like normal monocytes, THP-1 cells have shown to respond to mechanical stressors. For example, in models of atherosclerosis, biomechanical strain on THP-1 cells can induce expression of the class A scavenger receptor, an important lipoprotein receptor in atherogenesis (Yamamoto, Ikeda and Shimada, 2003). In addition, DNA microarray analysis has shown that cyclic mechanical strain in THP-1 cells induces expression of genes, some encoding for inflammatory markers such as IL-8 and IEX-1(Yamamoto, Ikeda and Shimada, 2003). In these cells, biomechanical deformation influences the degradation of extracellular matrix, monocyte differentiation, and promotion of atherosclerosis (Yamamoto, Ikeda and Shimada, 2003). In addition, as THP-1 cells differentiate they become adherent, a process which may be result in altered mechanosensitivity (Tsuchiya *et al.*, 1982; Schwende *et al.*, 1996).
In this study the cells were mechanically stimulated using 1000Hz vibrations. The vibrational 1000Hz stimulation has been used to study *in vitro* osteoblast differentiation with successful results (Nikukar *et al.*, 2013; Pemberton *et al.*, 2015; Tsimbouri, 2015; Robertson *et al.*, 2018), and in this study it was used to investigate any effect it may have on the differentiation of macrophages from THP-1 monocytes.
Overall, the experiments of this study were designed to give an insight into the differentiation process of THP-1 monocytes into macrophages under different stimulation parameters, compare between treatments, and look into mechanosensor mRNA expression.

Materials & Methods

THP-1 monocyte growth

THP-1 cells (ATCC® TIB-202™) were reconstituted from -80°C storage and allowed to recover for 2 weeks in cultures, splitting when confluency reached around $8x10^5$ cells/mL. The culture medium needed for cell growth was composed of RPMI-1640 with L-glutamine (Capricorn Scientific, RPMI-HA), 10 % Foetal Bovine Serum (FBS) (Gibco, A3160802) and 1% Antibiotic-Antimycotic 100X mix (Gibco, 15240062). The cells were cultured at 37°C, 5% CO_2 until ready for the experiments.

Experimental set up

The THP-1 cells were collected from T75 flasks (25mL suspension) and pelleted by centrifugation at 1500 rpm for 10 minutes. The experiment involved 4 replicates of untreated cells, 4 replicates of cells treated with 50nM 1,25-dihydroxyvitamin D3 (Sigma-Aldrich, D1530), 4 replicates of cells treated with 1000Hz vibrations (amplitude range of 30 - 60 nm), and 4 replicates of cells treated simultaneously with 50nM 1,25-dihydroxyvitamin D3 and 1000Hz stimulation. The cells underwent stimulation for 3 days (72 hours). The cell density per each replicate at the start of the experiment was $1.5x10^5$ cells/mL, in 1mL suspension plated on 24-well plates (Thermofisher Scientific, 142475). The experiments took place at 37°C, 5% CO_2, and 95% air incubator (LEEC 190D CO_2).

is medium changed each day with new D3?

Preparation of the vibrational device

Plates (24-well plates) which would be clamped on the bioreactor had magnet sheets (First4Magnets, D-F4MA43MHP) attached 48 hours before the start of the experiment, for better adhesion and removal of air pockets with time. In addition, the vibrational device (nicknamed Nanokicking bioreactor) was incubated at 37°C for 2 days prior to the start of experiments, which was the temperature at which the bioreactor displacements were calibrated. Incubation prior to the experiment was also useful for avoiding condensation upon immediate translocation of the bioreactor from room temperature to incubator environment. The experiments took place in fanless incubator LEEC 190D to avoid additional external vibrations. The bioreactors stability and generated vibrations were assessed using laser interferometry before the experiments. The platform of the bioreactor was generating vibrations of 1000Hz frequency and amplitude range 30-60 nm at the time of the experiments.

RNA extraction

The RNA was extracted separately for the suspension and adherent cells. Cell suspension was slowly removed and added to sterile RNA-free 1.5mL tubes. The cells in suspension were pelleted by centrifugation at 3000 rpm for 5 minutes. The supernatant was discarded and 1mL Trizol reagent (Invitrogen, AM9738) was added to homogenise the pellet. For the adherent cells, 1mL Trizol reagent was added directly on the wells. The lysed cells were homogenised using a 25g syringe. The RNA extraction from the lysed cells in Trizol solution was done by separating the aqueous phase after addition of 0.2mL chloroform and centrifugation at 13000 rpm for 15 minutes at 4°C. The RNA was washed with isopropanol and 75% ethanol and stored in 30µL of

nuclease-free water (Gibco, 10977035). Quantification of the RNA in ng/μL was done on Nanodrop 1000, using the RNA nucleic acid programme.

DNase treatment

The DNase treatment was performed following the protocol of DNA-free Kit (Thermofisher Scientific, AM1906), in order to degrade any genomic DNA that contaminated the RNA solutions during extraction. The maximum RNA concentration for each sample was 5μg per 50μL DNase reaction. Removal of genomic DNA contamination allowed efficient detection of amplification during the real-time PCR.

Complementary DNA synthesis

The synthesis of cDNA was done as instructed on the protocol of High-Capacity cDNA Reverse transcription Kit (Applied Biosystems, 4368814). The reaction was comprised of 10μL of 2X RT Mastermix and 10μL of purified RNA solution from the previous step. Reaction was started by warming at 25^{o}C for 10 minutes, followed by incubation at 37^{o}C for 2 hours for the synthesis of the cDNA, and termination of reaction at 85^{o}C for 5 minutes. The newly synthesised cDNA was stored at -20^{o}C until used for PCR reactions.

Real-time PCR

Real-time PCR was used to quantify gene expression in adherent and suspension THP-1 cells. The PCR amplifications were performed in 25μL reactions containing 12.5μL PowerUP SYBR Green Mastermix (Applied Biosystems^{TM}, A25742); 0.5μL Forward Primer and 0.5μL Reverse Primer for the respective genes, 1μL of cDNA and topped up to 25μL with nuclease free water (Gibco, 10977035).

The primer pair used for amplification of the housekeeper *RPL37A* were *RPL37A* forward 5'-ATTGAAATCAGCCAGCACGC-3' and *RPL37A* reverse 5'-AGGAACCACAGTGCCAGATCC-3'. The primer pair used for amplification of the housekeeper *ACTB* were *ACTB* forward 5'-ATTGCCGACAGGATGCAGAA-3' and *ACTB* reverse 5'-GCTGATCCACATCTGCTGGAA-3'. The primer pair used for amplification of *CD36* were *CD36* forward 5'-TCACTGCGACATGATTAATGGTACA-3' and *CD36* reverse 5'-ACGTCGGATTCAAATACAGCATAGAT-3'. The primer pair used for amplification of *CD14* were *CD14* forward 5'-ACGCCAGAACCTTGTGAGC-3' and *CD14* reverse 5'-GCATGGATCTCCACCTCTACTG-3'. The primer pair for amplification of *HLA-DRA* were *HLA-DRA* forward 5'-TAAGGCACATGGAGGTGATG-3' and *HLA-DRA* reverse 5'-GTACGGAGCAATCGAAGAGG-3'. The primer pair used for amplification of *HLA-DMB* were *HLA-DMB* forward 5'-CTCTCACAGCACCTCAACCA-3' and *HLA-DMB* reverse 5'-TAGAAGCCCCACACATAGCA-3'. The primer pair used for amplification of *PIEZO1* were *PIEZO1* forward 5'-CATCTTGGTGGTCTCCTCTGTCT-3' and *PIEZO1* reverse 5'-CTGGCATCCACATCCCTCTCATC-3'. The primer pair used for detection of *PKD1* were *PKD1* forward 5'-CGCCGCTTCACTAGCTTCGAC-3' and *PKD1* reverse 5'-ACGCTCCAGAGGGAGTCCAC-3'. The primer pair used for amplification of *PKD2* were *PKD2* forward 5'-GCGAGGTCTCTGGGGAAC-3' and *PKD2*

reverse 5'-TACACATGGAGCTCATCATGC-3'. The primer pair used for amplification of *NFAT2* were *NFAT2* forward 5'-CACTCCTGCTGCCTTACACA-3' and *NFAT2* reverse 5'-AAGATGCGAGCATGCGACTA-3'. The primer pair used for amplification of *TCF3* were *TCF3* forward 5'-TGACCTCCTGGACTTCAGC-3' and *TCF3* reverse 5'-ACCTGAACCTCCGAACTGC-3'. The primer pair used for amplification of *TCF4* were *TCF4* forward 5'-AGTGCGATGTTTTCACCTCC-3' and *TCF4* reverse 5'-CCTGAGCTACTTCTGTCTTC-3'. The primer pair used for the amplification of *TCF7L2* were *TCF7L2* forward 5'-CCGGGAAAGTTTGGAAGAAG-3' and *TCF7L2* reverse 5'-ACTGAAAATGGAGGGTTCGG-3'. The primer pair used for amplification of *LEF-1* were *LEF-1* forward 5'-GACAGTGACCTAATGCACGT-3' and *LEF-1* reverse 5'-CCACCTTCTGCCAAGAATCT-3'.

The primers for TCFs and LEF-1 transcription factors were generously provided by Dr. Robin Freeburn. Primers amplifying *PIEZO1* were designed using the NCBI primer design tool for the mRNA sequence NM_001142864.4, and primers amplifying *CD14* were designed similarly for the mRNA sequences NM_001174105.2 (CD14 mRNA transcript variant 4), NM_001040021.3 (CD14 mRNA transcript variant 2), NM_000591.4 (CD14 mRNA transcript variant 1) and NM_001174104.1 (CD14 mRNA transcript variant 3). The primers for *NFAT2* were obtained from Dagna *et al*. (Dagna, Pritchett and Lusso, 2013), primers for *HLA-DRA* and *HLA-DMB* were obtained from Ulbricht *et al*. (Ulbricht *et al.*, 2012), primers for *PKD1* and *PKD2* were obtained from Dalagiorgou *et al*. (Dalagiorgou *et al.*, 2013), and primers from *CD36*, *ACTB* and *RPL37A* were obtained from Maeß *et al.* (Maeß, Sendelbach and Lorkowski, 2010). The efficiency of primers taken from existing literature has been assessed in published papers (Fukuda, Mitsuoka and Schmid-Schönbein, 2004; Maeß, Sendelbach and Lorkowski, 2010; Ulbricht *et al.*, 2012; Dagna, Pritchett and Lusso, 2013; Dalagiorgou *et al.*, 2013). The primer efficiency was assessed prior to the experiments and was around 97% for all the investigated genes. Similar PCR efficiency for each primer is necessary for relative quantification using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). The PCR efficiency was also assessed by melt curve analysis. The collected C_T values were used for the $\Delta\Delta C_T$ relative quantification of expression, comparing the stimulated cells to the untreated controls. The ΔC_T was obtained by comparison of CTs of genes of interest to the mean C_T of two housekeeping genes *RPL37A* and *ACTB.* These housekeeping genes are considered to be the best for the analysis of RNA expression in THP-1 cells (Maeß, Sendelbach and Lorkowski, 2010).

Statistical analysis

The gene expression data are presented as mean of four replicates ± SEM, with little exception where some particular genes were not detected in all replicates. The analysis of statistical significance between the stimulated cells versus controls, and between each type of stimulation was done using unpaired T test with Welch's correction.

Statistical analysis was carried out using GraphPad Prism® version 6. P values <0.05 were accepted as significant.

Results

Regulation of genes encoding macrophage markers and antigen presenting molecules

Stimulation with vitamin D3, which also served as a positive control for the induction of differentiation, resulted in upregulation of the *CD14* and *CD36* mRNA in both adherent and suspension cells (**Error! Reference source not found.**, A; **Error! Reference source not found.**, A). The vitamin D3 downregulated the *HLA-DRA* expression in adherent and suspension cells (**Error! Reference source not found.**, A; **Error! Reference source not found.**, A). The mRNA of *HLA-DMB* was upregulated for vitamin D3 stimulation in suspension cells (**Error! Reference source not found.**, A). The *HLA-DMB* was not regulated in response to the vitamin D3 in the adherent cells (**Error! Reference source not found.**, A).

The 1000Hz stimulation caused upregulation of *CD36* and downregulation of *HLA-DMB* in suspension cells (**Error! Reference source not found.**, B), whereas in adherent cells it only downregulated the *HLA-DRA* (**Error! Reference source not found.**, B).

The combined stimulation induced upregulation of *CD14* and *CD36* in both adherent and suspension cells (**Error! Reference source not found.**, C; **Error! Reference source not found.**, C). The *HLA-DRA* was downregulated in both cell types compared to the respective unstimulated control (**Error! Reference source not found.**, C; **Error! Reference source not found.**, C), whereas *HLA-DMB* was upregulated in suspension cells (**Error! Reference source not found.**, C).

A comparison between the retreatments was performed for these genes in suspension and adherent cells (**Error! Reference source not found.**, A; **Error! Reference source not found.**, A).

CD14 was upregulated in response to the vitamin D3, as the mRNA levels were comparable to the cells stimulated by the vitamin only. Similarly, the upregulation of *CD36* in the adherent cells was only in response to the vitamin D3 in the combined treatment. In suspension cells undergoing the combined treatment, the 1000Hz stimulation weakened the upregulation of *CD36* by the vitamin D3, which was still higher than the upregulation caused by the 1000Hz vibrational stimulation alone. In the combined treatment, the 1000Hz vibrations also weakened the upregulation of *HLA-DMB* by the vitamin in the suspension cells. Interestingly, the *HLA-DRA* was downregulated from all treatments at the same level in the adherent cells.

Regulation of genes encoding transcription factor

The stimulation with vitamin D3 downregulated *NFAT2* and *TCF3* in adherent cells (**Error! Reference source not found.**, A). The *TCF4* and *LEF-1* were downregulated in both adherent and suspension cells stimulated with the vitamin (**Error! Reference source not found.**, A; **Error! Reference source not found.**, A). The *TCF7L2* mRNA

was upregulated in response to the stimulation with vitamin D3 in adherent and suspension cells (**Error! Reference source not found.**, A; **Error! Reference source not found.**, A).
The 1000Hz vibrational stimulation upregulated *TCF3* in both adherent and suspension cells compared to the respective controls (**Error! Reference source not found.**,B; **Error! Reference source not found.**, B). This type of stimulation also downregulated *NFAT2* in the adherent cells (**Error! Reference source not found.**, B). The mRNA expression of *TCF4*, *TCF7L2* and *LEF-1* were not affected by the vibrational stimulation (**Error! Reference source not found.**, B; **Error! Reference source not found.**, B).
The combined stimulation downregulated *NFAT2* in both adherent and suspension cells (**Error! Reference source not found.**, C; **Error! Reference source not found.**, C).
The *TCF3* mRNA was downregulated in the adherent cells (**Error! Reference source not found.**,C), but upregulated in the suspension cells (**Error! Reference source not found.**, C). The *TCF4* was downregulated in both cell types, and *TCF7L2* was upregulated in both cell types (**Error! Reference source not found.**, C; **Error! Reference source not found.**, C). The *LEF-1* was downregulated in the suspension cells (**Error! Reference source not found.**, C).
A comparison between the treatments was performed for these genes encoding transcription factors in the suspension (**Error! Reference source not found.**, B) and adherent cells (**Error! Reference source not found.**, B).
The *NFAT2* mRNA was downregulated in adherent cells for all the treatments, without difference between each other. In the suspension cells, the *NFAT2* was downregulated only for the combined stimulation.
In adherent cells, the *TCF3* mRNA was downregulated in response to vitamin D3 but upregulated for the 1000Hz stimulation. In the adherent cells, the vitamin cancelled the upregulating effect of the 1000Hz vibration and downregulated *TCF3*, at comparable levels to the cells stimulated with vitamin D3 only. However, in suspension cells, the *TCF3* upregulation was influenced by the 1000Hz vibrations, and the mRNA levels were comparable to the cells stimulated with the 1000Hz vibrations alone.
The *TCF4* mRNA was downregulated in response to vitamin D3 stimulation in both suspension and adherent cells. In the combined treatment, *TCF4* was influenced by the vitamin only. The 1000Hz did not have any influence on the expression of this gene neither alone or in combination with the vitamin. Similarly, the upregulation of *TCF7L2* mRNA was influenced only by the vitamin D3 in both adherent and suspension cells, with the 1000Hz stimulation having no effect on the cells when applied alone or in combination with the vitamin
The mRNA for *LEF-1* was downregulated in response to vitamin D3 stimulation. In the adherent cells little RNA was obtained for this gene, and no amplification was detected for the 1000Hz stimulation. This needs to be investigated in the future to explain whether the lack of amplification was due to very low transcripts levels in total RNA, or

because of some inhibitory effect that 1000Hz vibrations may have. In the suspension cells stimulated with the combined treatment, the 1000Hz weakened the downregulating effect of the vitamin D3, however the vitamin influenced the downregulation.

Regulation of genes encoding mechanosensors *PIEZO1, PKD1* and *PKD2*

The stimulation with the vitamin D3 resulted in upregulation of *PKD2* (*TRPP2*) mRNA in both adherent and suspension cells. The stimulation with the vitamin D3 alone did not affect the expression of *PIEZO1* or *PKD1* (*TRPP1*) (**Error! Reference source not found.**, A **Error! Reference source not found.**, A).
The 1000Hz vibrational stimulation resulted in upregulation of *PIEZO1* mRNA in both adherent and suspension cells. In the adherent cells, the stimulation downregulated *PKD2* mRNA. The vibrational stimulation did not affect *PKD1* expression (**Error! Reference source not found.**, B **Error! Reference source not found.**, B).
The combined treatment resulted in the upregulation of *PKD2* mRNA in both adherent and suspension cells. *PIEZO1* and *PKD1* were not regulated in cells stimulated with the combined treatment (**Error! Reference source not found.**, C **Error! Reference source not found.**, C).
A comparison between treatments was performed for these genes encoding mechnosensors in the suspension (**Error! Reference source not found.**, C) and adherent cells (**Error! Reference source not found.**, C).
The expression of *PKD2* was affected only by the vitamin D3, which also cancelled the downregulation effect of the 1000Hz in the adherent cells stimulated with the combined treatment.
The *PIEZO1* upregulation occurred only in response to the stimulation with 1000Hz vibrations, but in the combined treatment the vitamin cancelled the upregulating effect of the vibrational stimulation
The expression of *PKD1* mRNA was not affected by any of the stimulation methods.

Discussion

TCF/LEF pathway and gene nomenclature

TCF/LEF pathway plays roles in monocyte and macrophage differentiation (Thiele *et al.*, 2001). It must be mentioned that some confusion exists about the nomenclature of the TCFs. The mammalian TCF/LEF family comprises of four nuclear factors designated TCF7, LEF1, TCF7L1, and TCF7L2, which are also known as TCF1, LEF1, TCF3, and TCF4, respectively (Hrckulak *et al.*, 2016). Confusion also exists between the nomenclature of genes and the corresponding products. For example, a gene called *TCF3* (NCBI gene ID: 6929), also known as *E2A*, encodes a product that is different from TCF3 encoded from *TCF7L1* (NCBI Gene ID: 83439). Similarly, *TCF4* (NCBI gene ID: 6925), encodes for TCF4 which is a different protein from the

TCF4 encoded from *TCF7L2* (NCBI Gene ID: 6934). In this experiment, the mRNA investigated belongs to genes *TCF3* (E2A), *TCF4* (E2-2), *TCF7L2* and *LEF-1*, with the last two investigated in the context of WNT canonical pathways in monocyte-derived macrophages (Malsin *et al.*, 2019). The pathways which involve *TCF3* and *TCF4* gene products can be complex and are not elucidated in context of monocyte to macrophage differentiation.

Vitamin D3 induced macrophage differentiation and downregulated *HLA-DRA*

The vitamin D3 has shown to target multiple monocyte genes and promote monocyte differentiation into macrophages (Nurminen, Seuter and Carlberg, 2019). Similarly, this study demonstrated that vitamin D3 stimulation induced differentiation of THP-1 monocytes into macrophages, when looking at transcriptional regulation of *CD14*, *CD36* and transcription factors *TCF7L2* (encoding TCF4) and *LEF-1*.
The stimulation with vitamin D3 resulted in upregulation of the *CD14* and *CD36* mRNA in both adherent and suspension cells. This pattern of regulation for these two genes was expected to occur during macrophage differentiation from monocytes (Zhang *et al.*, 1994; Maeß, Sendelbach and Lorkowski, 2010). The CD14 is an important marker of the THP-1 differentiation into macrophages which upregulates strongly upon vitamin D3 stimulation (Schwende *et al.*, 1996; Gocek *et al.*, 2012), as was also observed in this study. Furthermore, *CD14* and *CD36* are primary target genes for vitamin D3 in THP-1 monocytes (Nurminen, Seuter and Carlberg, 2019). The CD14 and CD36 are proteins involved in macrophage functions. CD14 cooperates with other proteins to mediate the innate immune response to bacterial lipopolysaccharide, whereas CD36 is a scavenger receptor involved in phagocytosis (Woo *et al.*, 2016).
The vitamin D3 downregulated the expression of *HLA-DRA* in differentiating THP-1 cells. Downregulation of the HLA-DR protein has been observed in primary monocytes treated with vitamin D3 (Tokuda and Levy, 1996), as well as in dendritic cells (Ferreira *et al.*, 2015). In dendritic cells, the downregulation of HLA-DR has been suggested to be part of tolerance processes induced by vitamin D3 signalling (Ferreira *et al.*, 2015). The *HLA-DRA* downregulation in this study was an interesting observation which agreed with the observed immunomodulatory effects of the vitamin D (Yang *et al.*, 2013).
In addition, the vitamin D3 stimulation upregulated the mRNA of *HLA-DMB* in suspension cells. This molecule is important for antigen loading of the MHC class II by removal of CLIP from HLA-DR (Riberdy *et al.*, 1992; Sloan *et al.*, 1995). In one study, HIV-infected THP-1 monocytes had loss of mRNA for *HLA-DR*, but the mRNAs for *HLA-DM* continued to be transcribed, showing that genes may have non-corresponding expression patterns (Shao and Sperber, 2002), similar to what was observed in this study.
This study also identified an inverse relationship between *TCF7L2* and *LEF-1* mRNA regulation upon vitamin D3-induced macrophage differentiation. The *TCF7L2* (encoding TCF4) in combination with β-catenin forms a complex that regulates expression of

genes in monocytes and it is thus involved in the differentiation process (Thiele *et al.*, 2001; Tickenbrock, 2006; Malsin *et al.*, 2019), whereas LEF-1 facilitates nuclear localisation of β-catenin and enhances proliferation in acute myeloid leukaemia cells, including THP-1 cells (Morgan *et al.*, 2019). Therefore, the downregulation of *LEF-1* and the upregulation of *TCF7L2* could indicate decreased proliferation and increased differentiation as THP-1 monocytes become macrophages (Schwende *et al.*, 1996; Thiele *et al.*, 2001; Morgan *et al.*, 2019). The inverse relationship of *TCF7L2* and *LEF-1* has also been related to shifts in differentiation and proliferation states in other cancer cells (Kriegl *et al.*, 2010; Eichhoff *et al.*, 2011). This pattern of regulation for these two genes can be signature of THP-1 monocyte to macrophage differentiation.

Another transcription factor downregulated in adherent cells in response to the vitamin D3 was *NFAT2*. The NFATs are important transcription factors for production of proinflammatory cytokines in T and B cells (Macian, 2005), but their roles are not only limited to the adaptive immune cells. It has been showed that the NFATs are required for Toll-like receptor (TLR)-initiated innate immune responses in bone marrow-derived macrophages (Minematsu *et al.*, 2011). In THP-1 monocytes *in vitro*, the NFAT2 has shown to inhibit the release of high mobility protein box-1 (HMGB1) (Zhao Q *et al.*, 2016), a proinflammatory protein with roles in inflammation and autoimmunity (Magna and Pisetsky, 2014). The suppression of NFAT2 expression by siRNA has resulted in increased HMGB1 in the supernatant of cells (Zhao Q *et al.*, 2016). In T cells, 1,25-dihydroxyvitamin D3 and its receptor complex (VDR-RXR) have shown to inhibit NFAT activity (Wöbke, Sorg and Steinhilber, 2014), but its effect on monocytes and *NFAT2* mRNA are not known. In this study, the downregulation of *NFAT2* mRNA in the adherent cells, which are considered to be in a more advanced stage of differentiation than the suspension cells (Tsuchiya *et al.*, 1982; Schwende *et al.*, 1996), could be related to the production of proinflammatory proteins after the maturation of the monocytes into macrophages.

The vitamin D3 stimulation also downregulated *TCF3* (encoding E2A) in adherent cells, and *TCF4* (encoding E2-2) in both cell types compared to the respective controls. The roles of the products of these genes are not known in monocyte biology and macrophage differentiation, but as demonstrated in this study they are regulatable upon vitamin D3 stimulation.

The stimulation with vitamin D3 had no effect on the regulation of *PIEZO1* or *PKD1*, but it upregulated *PKD2* (*TRPP2*) mRNA in both suspension and adhesion cells. The roles of polycystin 2 (product of *PKD2*) are not known in THP-1 monocytes, but this study demonstrated that the *PKD2* mRNA upregulation is signature of vitamin D3-induced differentiation.

Monocyte responses to 1000Hz vibrational stimulation

The THP-1 monocytes are responsive to mechanical stressors. Biomechanical strain on THP-1 cells can induce expression of the class A scavenger receptor, degradation of extracellular matrix, monocyte differentiation, and promotion of atherosclerosis (Yamamoto, Ikeda and Shimada, 2003). In addition, DNA microarray analysis has shown that cyclic mechanical strain in THP-1 cells induces expression of genes, some encoding for inflammatory markers such as IL-8 and IEX-1 (Yamamoto, Ikeda and Shimada, 2003). Furthermore, upon differentiation, THP-1 cells become adherent (Tsuchiya *et al.*, 1982; Schwende *et al.*, 1996), which may result in altered mechanosensitivity. This study used vibrations as means of mechanical stimulation of these cells in order to study their mechanosensitivity.

The vibrational 1000Hz stimulation resulted in upregulation of *PIEZO1* transcripts in both suspension and adhesion cells. PIEZO1 channels are considered professional mechanosensory proteins, capable of sensing and converting mechanical stimuli (Zhong *et al.*, 2018). Little is known about the mechanosensory roles of these channels in monocytes and macrophages. PIEZO1 has shown to signal in response to cyclical hydrostatic pressure, resulting in HIF1α stabilization and secretion of molecules, such as endothelin-1 (EDN1), and neutrophil chemoattractant CXCL2 (Solis *et al.*, 2019). The PIEZO1 signalling to the cyclical pressure has induced inflammation and infiltration of monocytes, which recruit neutrophils in order to clear pulmonary *Pseudomonas aeruginosa* infection via EDN1 (Solis *et al.*, 2019). In this study, we demonstrate that THP-1 cells regulated PIEZO1 mRNA in response to 1000Hz vibrational stimulation, but the biological significance of such regulation remains to be elucidated.

The 1000Hz stimulation also caused *HLA-DRA* downregulation in adherent cells similar to vitamin D3, but when combined with the vitamin it did not show any synergetic effect. Another gene which was upregulated during the stimulation with 1000Hz vibrations, was *TCF3*. This gene was upregulated in both suspension and adherent cells, but the role of this gene and its products are not known in monocytes. The 1000Hz vibrations downregulated the *NFAT2* mRNA at the same levels as the vitamin D3 in adherent cells, and just like the vitamin it did not regulate this gene in suspension cells.

The vibrational stimulation had not effect on the regulation of other transcription factors such as *TCF4*, *TCF7L2* and *LEF-1,* which were influenced by the vitamin D3 only.

The upregulation of *PIEZO1* and *TCF3* upon the application of the 1000Hz stimulation was interesting, but it was not associated with macrophage differentiation, because there was no transcriptional regulation for genes such as *CD14*, *TCF7L2* and *LEF-1* which would indicate transition from monocytes to macrophages. The *CD36* was upregulated for the 1000Hz stimulation in suspension cells. However, in adherent cells, which are considered to be in a more advanced stage of differentiation (Tsuchiya *et al.*, 1982; Schwende *et al.*, 1996), the *CD36* mRNA levels were comparable to the unstimulated controls.

The effects of the combined treatment on gene expression and comparison to vitamin D3 and 1000Hz vibrations

In the combined treatment, the differentiation into macrophages took place but it was influenced mostly by the vitamin D3.
The upregulation of *CD14* in suspension and adherent cells undergoing the combined treatment was comparable to cells stimulated with vitamin D3 only. The *CD36* mRNA was upregulated in the adherent cells at comparable level to the cells stimulated with vitamin D3 only. However, in the suspension cells the 1000Hz had slightly weakened the upregulation of *CD36* by the vitamin D3. The combination of both stimuli resulted in mRNA expression lower than the stimulation with the vitamin, but higher that the stimulation with the 1000Hz, hence it could be said that the 1000Hz weakened the upregulating effect of the vitamin.
Even though when applied in isolation the 1000Hz stimulation caused *HLA-DRA* downregulation in adherent cells at similar levels to vitamin D3, in the stimulation it did not show any synergetic effect. The downregulation of *HLA-DRA* in suspension cells undergoing the combined treatment was comparable to the cells stimulated with the vitamin D3 only, showing that in the combined treatment this gene was influenced only to the vitamin.
In the suspension cells undergoing the combined treatment, the 1000Hz weakened the upregulation of *HLA-DMB* by the vitamin. When applied in isolation the 1000Hz vibrations downregulated HLA-DMB, however, in the combined treatment the vitamin overshadowed the effect of the vibrational stimulus and caused upregulation.
The combined treatment downregulated *NFAT2* at comparable levels to both the vitamin D3 and 1000Hz treatments when applied alone in the adherent cells. However, in the suspension cells, the 1000Hz and the vitamin D3 may have synergistically caused the downregulation of *NFAT2* in suspension cells, because the vitamin and the 1000Hz did not regulate this gene when applied in isolation.
The regulation of *TCF4* and *TCF7L2* in the cells stimulated with the combined treatment was comparable to the cells stimulated with vitamin D3, and the 1000Hz stimulation had no effect on these genes in the combined treatment, similar to when it was applied in isolation. The 1000Hz vibrations however, weakened the downregulating effect that the vitamin had on the mRNA encoding *LEF-1* in the suspension cell. In the adherent cells, the *LEF-1* mRNA in stimulated with the combined treatment was comparable to the unstimulated controls, but since the mRNA for this gene was not detected in cell stimulated with vibrations only, comparison could not take place.
In the presence of the vitamin D3, the effect of 1000Hz stimulation on the regulation of PIEZO1 was cancelled in both adherent and suspension cells. Furthermore, in adherent cells, the vitamin D3 cancelled the upregulating effect of 1000Hz on the *TCF3* and downregulated the gene. However, in suspension cells the 1000Hz stimulation

continued to upregulate *TCF3* even in the presence of the vitamin. This was the only case in which the effects of 1000Hz strongly influenced the expression pattern of a gene in the presence of the vitamin.

Conclusions

This study demonstrated that the stimulation with 50nM vitamin D3 for 3 days drives macrophage differentiation, as was determined by upregulation of *CD14*, *CD36* and *TCF7L2*, and downregulation of *LEF-1*. The differentiation in response to the vitamin D3 was accompanied by downregulation of *HLA-DRA* and upregulation of *PKD2* mRNA. Other genes that were regulated during vitamin D3-induced macrophage differentiation included *TCF3* and *TCF4* in both suspension and adherent cells, and *NFAT2* in adherent cells. The upregulation of the non-selective cation channel *PKD2* mRNA could suggest important roles that this mechanosensitive protein has in THP-1 differentiation. The downregulation of *HLA-DRA* could also be indicative of predisposition to M2-biased phenotype.

The vibrational stimulation which was used for the mechanical stimulation of cells did not induce the macrophage differentiation process because there was no transcriptional regulation of *CD14* and *TCF/LEF* transcription factors. However, the 1000Hz vibrations influenced upregulation of *PIEZO1* and *TCF3* in both adherent and suspension cells. Furthermore, in adherent cells, the vibrational stimulation downregulated *NFAT2* and *HLA-DRA* at comparable levels to the vitamin D3 stimulated adherent cells. This was indicative that while the 1000Hz vibrations did not induce differentiation, they induced regulation of genes in the THP-1 cells. However, the biological importance of such response remains to be elucidated.

In the combined treatment, the 1000Hz interfered with the regulation of the genes by the vitamin D3 but without changing the regulation patten of these genes. The only exception was *TCF3* in suspension cells stimulated with the combined treatment, which was upregulated by the 1000Hz vibrations against the downregulating influence of the vitamin D3. The biological importance of such interference remains to be elucidated. However, the mRNA regulation patterns for the majority of the genes in the combined treatment were in response to the vitamin D3 stimulation.

Furthermore, the influence of the 1000Hz stimulus in the presence of the vitamin D3 was cancelled (e.g. for *PIEZO1* in both cell types), overshadowed (e.g. for *CD36* in suspension cells), or cancelled and reversed (e.g. *PKD2* in adherent cells). This can

have implication for the medicinal application of the 1000Hz (nano-scale amplitude) vibrations, because in inflamed tissues rich in chemical signals such as cytokines and chemokines, the cells may lose the ability to sense and respond to such mechanical stimulus.

Further work in necessary to assess the reproducibility of the observations of this study, especially in response to the 1000Hz vibrational stimulation. This study had limitation because of the technology, which was not provided for repeated runs and further work. Larger number of replicates and expanded time-points are recommended for future work from the authors of this report.

Overall, this study presents experimental results indicating that the vibrational mechanical forces can be sensed by THP-1 monocytes, but that the chemical ligands such as vitamin D3 remain superior for the induction of macrophage differentiation.

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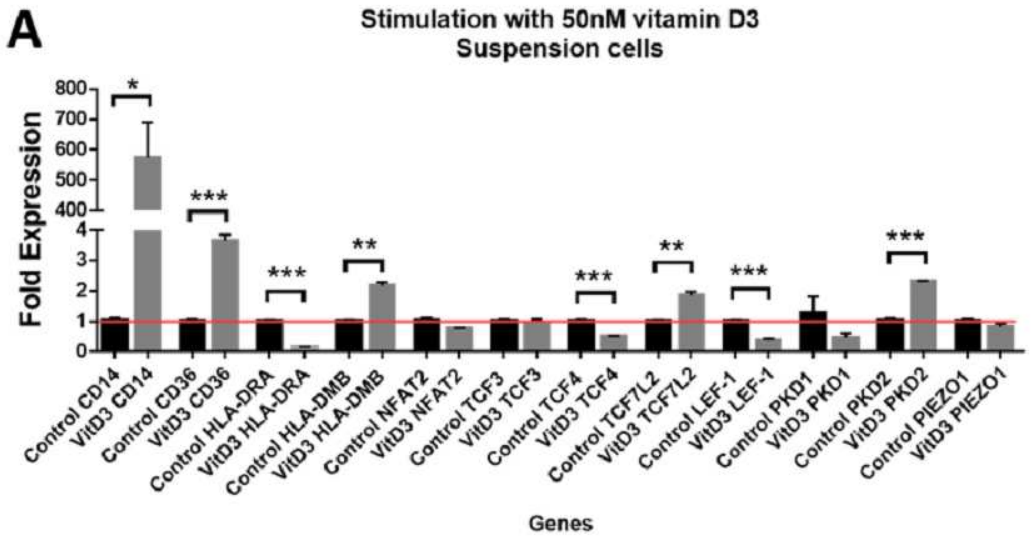
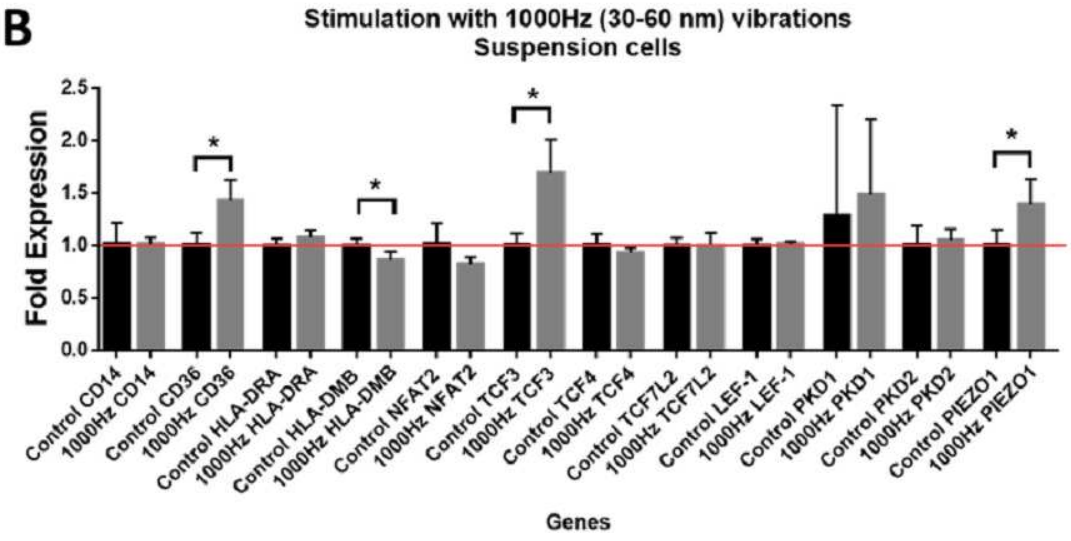
Figure 1

Gene expression in response to different stimulations in suspension THP-1 cells, compared to the unstimulated suspension THP-1 cells.

Data presented as mean of 4 replicates ± SEM. Statistical analysis between stimulated and control values was assessed by T test with Welch's correction. P values less that 0.05 were considered statistically significant.


A
Stimulation with 50nM vitamin D3
Suspension cells
Fold Expression
Control CD14
VitD3 CD14
Control CD36
VitD3 CD36
Control HLA-DRA
VitD3 HLA-DRA
Control HLA-DMB
VitD3 HLA-DMB
Control NFAT2
VitD3 NFAT2
Control TCF3
VitD3 TCF3
Control TCF4
VitD3 TCF4
Control TCF7L2
VitD3 TCF7L2
Control LEF-1
VitD3 LEF-1
Control PKD1
VitD3 PKD1
Control PKD2
VitD3 PKD2
Control PIEZO1
VitD3 PIEZO1
Genes


B
Stimulation with 1000Hz (30-60 nm) vibrations
Suspension cells
Fold Expression
Control CD14
1000Hz CD14
Control CD36
1000Hz CD36
Control HLA-DRA
1000Hz HLA-DRA
Control HLA-DMB
1000Hz HLA-DMB
Control NFAT2
1000Hz NFAT2
Control TCF3
1000Hz TCF3
Control TCF4
1000Hz TCF4
Control TCF7L2
1000Hz TCF7L2
Control LEF-1
1000Hz LEF-1
Control PKD1
1000Hz PKD1
Control PKD2
1000Hz PKD2
Control PIEZO1
1000Hz PIEZO1
Genes

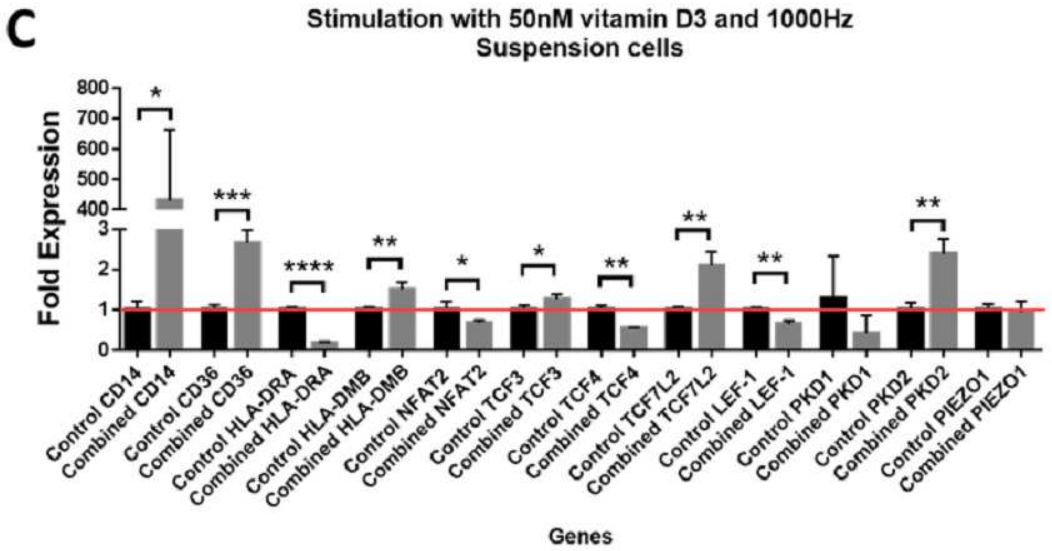
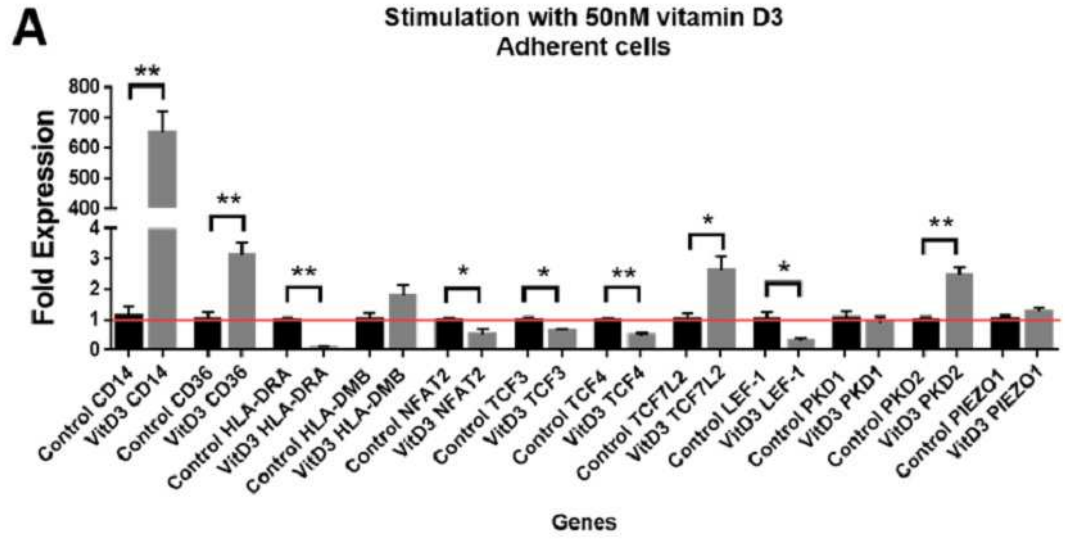
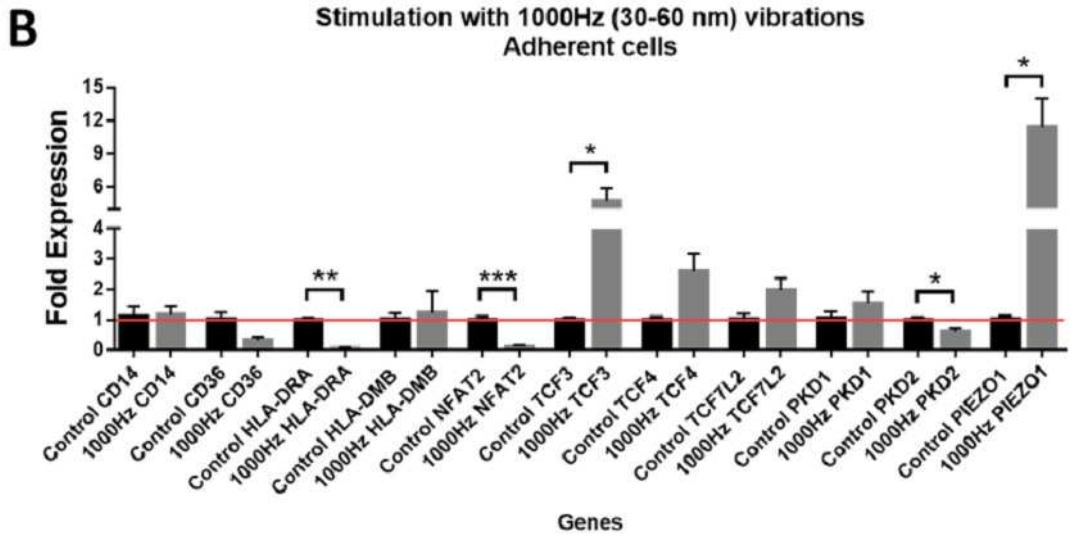

C
Stimulation with 50nM vitamin D3 and 1000Hz
Suspension cells
Fold Expression
Control CD14
Combined CD14
Control CD36
Combined CD36
Control HLA-DRA
Combined HLA-DRA
Control HLA-DMB
Combined HLA-DMB
Control NFAT2
Combined NFAT2
Control TCF3
Combined TCF3
Control TCF4
Combined TCF4
Control TCF7L2
Combined TCF7L2
Control LEF-1
Combined LEF-1
Control PKD1
Combined PKD1
Control PKD2
Combined PKD2
Control PIEZO1
Combined PIEZO1
Genes

Figure 2

Gene expression in response to different stimulations in adherent THP-1 cells, compared to the unstimulated adherent THP-1 cells.

Data presented as mean of 4 replicates ± SEM. Statistical analysis between stimulated and control values was assessed by T test with Welch's correction. P values less that 0.05 were considered statistically significant.


A
Stimulation with 50nM vitamin D3
Adherent cells
Fold Expression
Control CD14
VitD3 CD14
Control CD36
VitD3 CD36
Control HLA-DRA
VitD3 HLA-DRA
Control HLA-DMB
VitD3 HLA-DMB
Control NFAT2
VitD3 NFAT2
Control TCF3
VitD3 TCF3
Control TCF4
VitD3 TCF4
Control TCF7L2
VitD3 TCF7L2
Control LEF-1
VitD3 LEF-1
Control PKD1
VitD3 PKD1
Control PKD2
VitD3 PKD2
Control PIEZO1
VitD3 PIEZO1
Genes


B
Stimulation with 1000Hz (30-60 nm) vibrations
Adherent cells
Fold Expression
Control CD14
1000Hz CD14
Control CD36
1000Hz CD36
Control HLA-DRA
1000Hz HLA-DRA
Control HLA-DMB
1000Hz HLA-DMB
Control NFAT2
1000Hz NFAT2
Control TCF3
1000Hz TCF3
Control TCF4
1000Hz TCF4
Control TCF7L2
1000Hz TCF7L2
Control PKD1
1000Hz PKD1
Control PKD2
1000Hz PKD2
Control PIEZO1
1000Hz PIEZO1
Genes

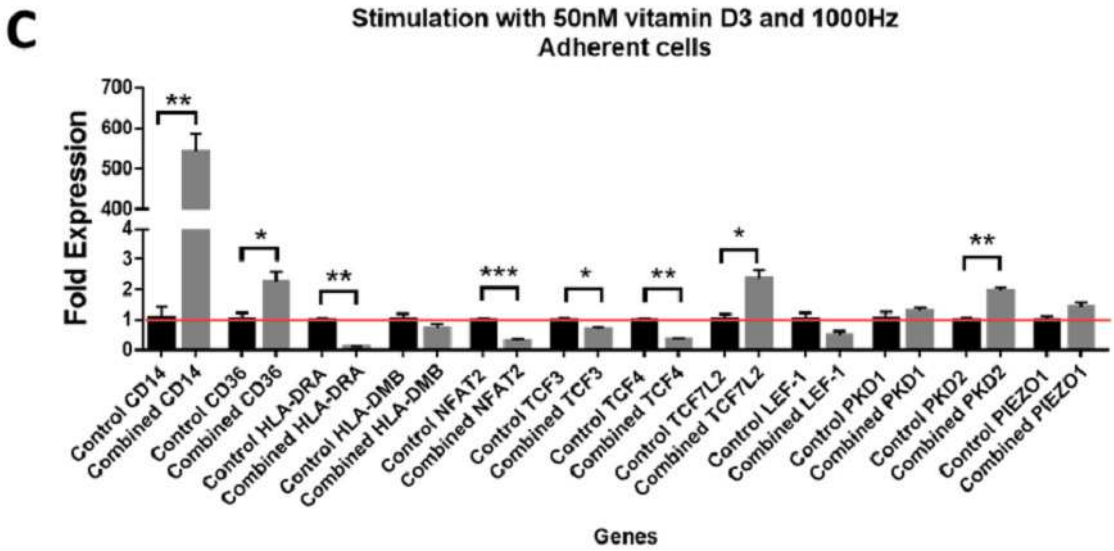
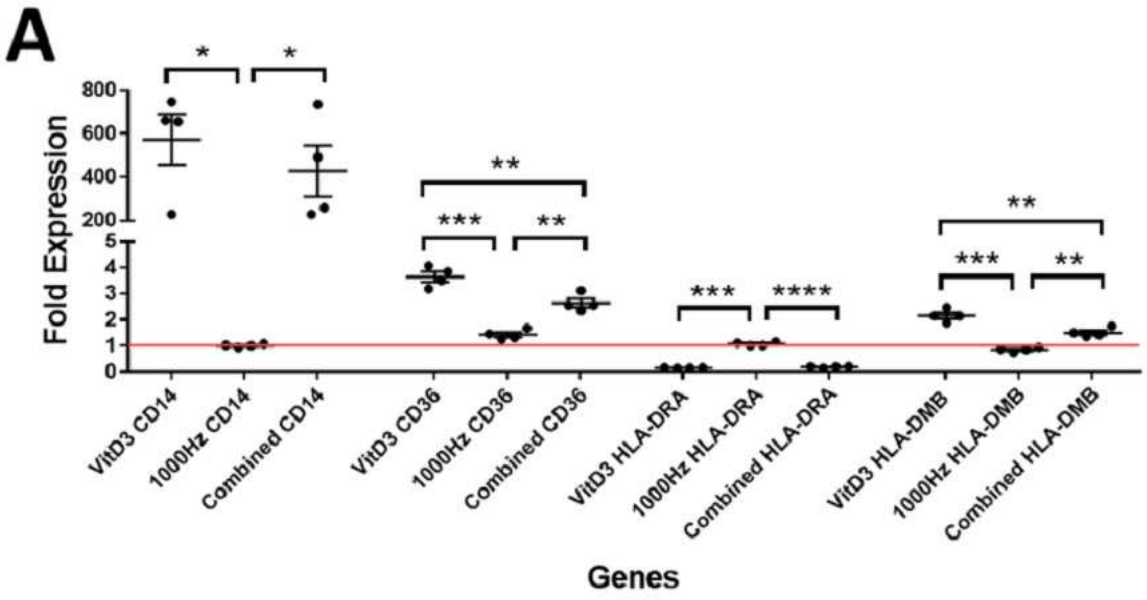
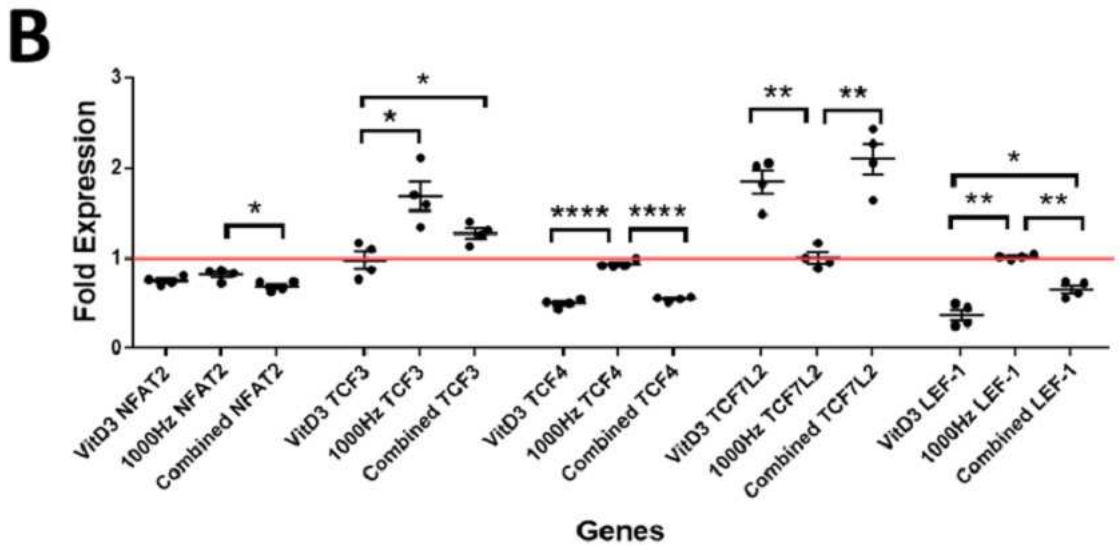

C
Stimulation with 50nM vitamin D3 and 1000Hz
Adherent cells
Fold Expression
Control CD14
Combined CD14
Control CD36
Combined CD36
Control HLA-DRA
Combined HLA-DRA
Control HLA-DMB
Combined HLA-DMB
Control NFAT2
Combined NFAT2
Control TCF3
Combined TCF3
Control TCF4
Combined TCF4
Control TCF7L2
Combined TCF7L2
Control LEF-1
Combined LEF-1
Control PKD1
Combined PKD1
Control PKD2
Combined PKD2
Control PIEZO1
Combined PIEZO1
Genes

Figure 3

Comparison of fold change values for different genes between the treatments in stimulated THP-1 suspension cells.

Each treatment values were compared to the others using T test with Welch's correction. P values less than 0.05 were considered statistically significant. Genes investigated encode for markers of macrophage differentiation (A), transcription factors (B), and mechanosensors (C).


A
Fold Expression
800
600
400
200
5
4
3
2
1
0
*
*
**
***
**
***
****
**
***
**
VitD3 CD14
1000Hz CD14
Combined CD14
VitD3 CD36
1000Hz CD36
Combined CD36
VitD3 HLA-DRA
1000Hz HLA-DRA
Combined HLA-DRA
VitD3 HLA-DMB
1000Hz HLA-DMB
Combined HLA-DMB
Genes


B
Fold Expression
3
2
1
0
*
*
*
*
****
****
**
**
*
**
**
VitD3 NFAT2
1000Hz NFAT2
Combined NFAT2
VitD3 TCF3
1000Hz TCF3
Combined TCF3
VitD3 TCF4
1000Hz TCF4
Combined TCF4
VitD3 TCF7L2
1000Hz TCF7L2
Combined TCF7L2
VitD3 LEF-1
1000Hz LEF-1
Combined LEF-1
Genes

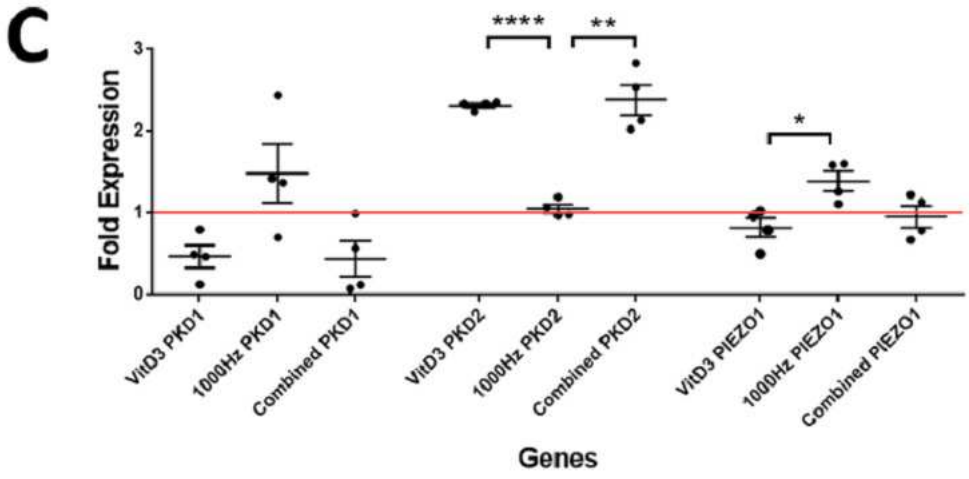
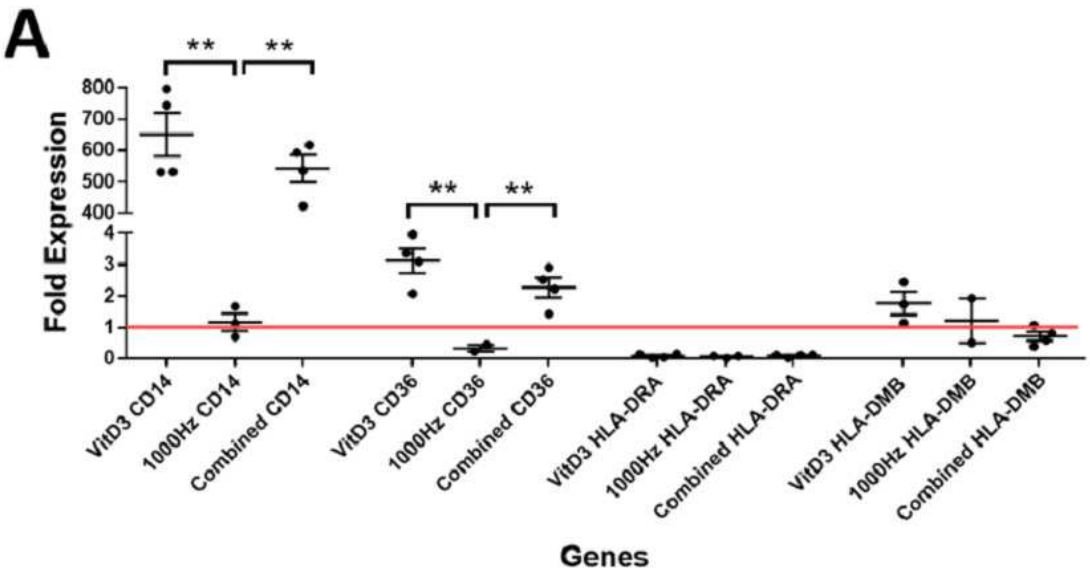
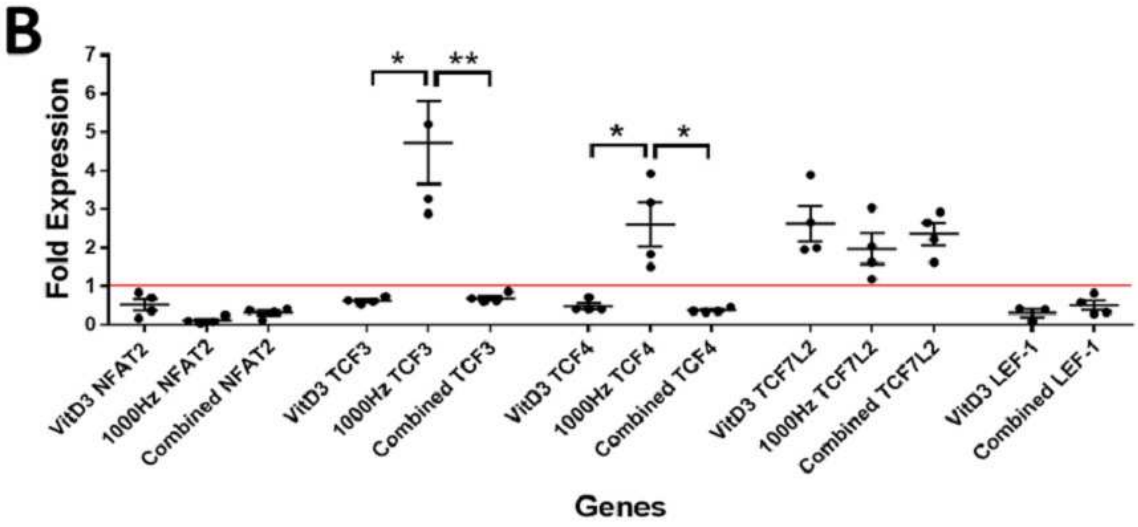

C
Fold Expression
3
2
1
0
****
**
*
VitD3 PKD1
1000Hz PKD1
Combined PKD1
VitD3 PKD2
1000Hz PKD2
Combined PKD2
VitD3 PIEZO1
1000Hz PIEZO1
Combined PIEZO1
Genes

Figure 4

Comparison of fold change values for different genes between the treatments in stimulated THP-1 suspension cells.

Each treatment values were compared to the others using T test with Welch's correction. P values less than 0.05 were considered statistically significant. Genes investigated encode for markers of macrophage differentiation (A), transcription factors (B), and mechanosensors (C).


A
Fold Expression
800
700
600
500
400
4
3
2
1
0
**
**
**
**
VitD3 CD14
1000Hz CD14
Combined CD14
VitD3 CD36
1000Hz CD36
Combined CD36
VitD3 HLA-DRA
1000Hz HLA-DRA
Combined HLA-DRA
VitD3 HLA-DMB
1000Hz HLA-DMB
Combined HLA-DMB
Genes


B
Fold Expression
7
6
5
4
3
2
1
0
*
**
*
*
VitD3 NFAT2
1000Hz NFAT2
Combined NFAT2
VitD3 TCF3
1000Hz TCF3
Combined TCF3
VitD3 TCF4
1000Hz TCF4
Combined TCF4
VitD3 TCF7L2
1000Hz TCF7L2
Combined TCF7L2
VitD3 LEF-1
Combined LEF-1
Genes

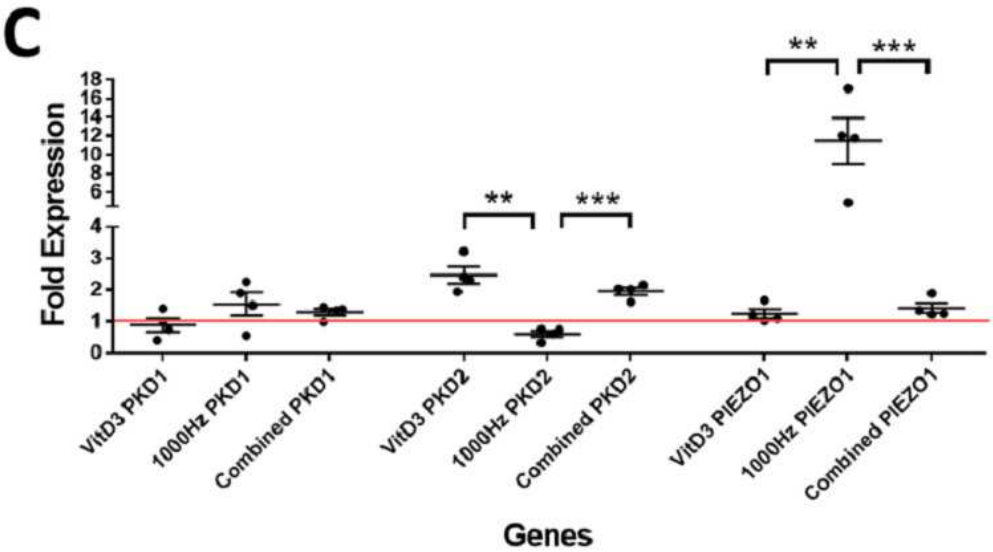

C
Fold Expression
18
16
14
12
10
8
6
4
3
2
1
0
**
***
**
***
VitD3 PKD1
1000Hz PKD1
Combined PKD1
VitD3 PKD2
1000Hz PKD2
Combined PKD2
VitD3 PIEZO1
1000Hz PIEZO1
Combined PIEZO1
Genes

Table 1(on next page)

Gene expression in stimulated suspension THP-1 cells compared to the unstimulated suspension cells.

Statistical analysis was performed using T test with Welch's correction. Fold change values higher than 1 indicate upregulation, whereas values between 0 and 1 indicate downregulation of mRNA transcripts in stimulated cells.

THP-1 cells in suspension				
Stimulation	**mRNA**	**Roles**	**Fold change (2^-ΔΔCT) Stimulated cells vs Control**	**P value**
50 nM 1,25(OH)2 D3 (72 hours)	***CD14***	**Macrophage marker**	**573.92**	**0.0161**
	CD36	**Macrophage marker**	**3.66**	**0.0004**
	HLA-DRA	**Antigen presentation**	**0.16**	**0.0001**
	HLA-DMB	**Antigen presentation**	**2.17**	**0.0016**
	PKD2	**Mechanosensory non-selective cation channel**	**2.32**	**0.0004**
	TCF4	**Transcription factor** (unknown roles in macrophages)	**0.5**	**0.001**
	TCF7L2	**Transcription factor** (proliferation and differentiation)	**1.85**	**0.0052**
	LEF-1	**Transcription factor** (proliferation and differentiation)	**0.37**	**0.0005**
Vibrations 1000Hz (30 - 60 nm) (72 hours)	***CD36***	**Macrophage marker**	**1.43**	**0.013**
	HLA-DMB	**Antigen presentation**	**0.86**	**0.0404**
	PIEZO1	**Mechanosensory channel**	**1.39**	**0.0441**
	TCF3	**Transcription factor** (unknown roles in macrophages)	**1.69**	**0.0182**
50 nM 1,25(OH)2 D3 + Vibrations 1000Hz (30 - 60 nm) (72 hours)	***CD14***	**Macrophage marker**	**428.9**	**0.0359**
	CD36	**Macrophage marker**	**2.66**	**0.0009**
	HLA-DRA	**Antigen presentation**	**0.19**	**< 0.0001**
	HLA-DMB	**Antigen presentation**	**1.51**	**0.0071**
	PKD2	**Mechanosensory non-selective cation channel**	**2.38**	**0.0021**
	TCF3	**Transcription factor** (unknown roles in macrophages)	**1.27**	**0.0142**
	TCF4	**Transcription factor** (unknown roles in macrophages)	**0.55**	**0.0026**
	NFAT2	**Transcription factor** (undefined roles in macrophages)	**0.69**	**0.0434**
	TCF7L2	**Transcription factor** (proliferation and differentiation)	**2.1**	**0.0063**
	LEF-1	**Transcription factor** (proliferation and differentiation)	**0.66**	**0.0011**

Table 2(on next page)

Gene expression in adherent THP-1 cells compared to the unstimulated adherent cells.

Statistical analysis was performed using T test with Welch's correction. Fold change values higher than 1 indicate upregulation, whereas values between 0 and 1 indicate downregulation of mRNA transcripts in stimulated cells.

THP-1 cells adhered				
Stimulation	**mRNA**	**Roles**	**Fold change (2^-ΔΔCT) Stimulated cells vs Control**	**P value**
50 nM 1,25(OH)2 D3 (72 hours)	***CD14***	**Macrophage marker**	**650.9**	**0.0026**
	CD36	**Macrophage marker**	**3.13**	**0.0073**
	HLA-DRA	**Antigen presentation**	**0.09**	**0.0011**
	PKD2	**Mechanosensory non-selective cation channel**	**2.47**	**0.0096**
	TCF3	**Transcription factor** (unknown roles in macrophages)	**0.63**	**0.0134**
	TCF4	**Transcription factor** (unknown roles in macrophages)	**0.48**	**0.0032**
	NFAT2	**Transcription factor** (undefined roles in macrophages)	**0.52**	**0.0458**
	TCF7L2	**Transcription factor** (proliferation and differentiation)	**2.63**	**0.0313**
	LEF-1	**Transcription factor** (proliferation and differentiation)	**0.3**	**0.0491**
Vibrations 1000Hz (30 - 60 nm) (72 hours)	***HLA-DRA***	**Antigen presentation**	**0.07**	**0.0022**
	PIEZO1	**Mechanosensory channel**	**11.44**	**0.0247**
	PKD2	**Mechanosensory non-selective cation channel**	**0.6**	**0.0236**
	NFAT2	**Transcription factor** (undefined roles in macrophages)	**0.12**	**0.0004**
	TCF3	**Transcription factor** (unknown roles in macrophages)	**4.73**	**0.04**
50 nM 1,25(OH)2 D3 + Vibrations 1000Hz (30 - 60 nm) (72 hours)	***CD14***	**Macrophage marker**	**542.09**	**0.0011**
	CD36	**Macrophage marker**	**2.27**	**0.0227**
	HLA-DRA	**Antigen presentation**	**0.09**	**0.002**
	PKD2	**Mechanosensory non-selective cation channel**	**1.95**	**0.0013**
	TCF3	**Transcription factor** (unknown roles in macrophages)	**0.69**	**0.0232**
	TCF4	**Transcription factor** (unknown roles in macrophages)	**0.37**	**0.0016**
	NFAT2	**Transcription factor** (undefined roles in macrophages)	**0.31**	**0.0006**
	TCF7L2	**Transcription factor** (proliferation and differentiation)	**2.36**	**0.0116**

Table 3(on next page)

Comparison of THP-1 gene expression between different treatments.

The arrows indicate upregulation or downregulation of the genes when comparing the different stimuli. Statistical analysis was performed using T test with Welch's correction

mRNA	50nM Vitamin D3 vs 1000Hz		50nM Vitamin D3 vs 50nM Vitamin D3+1000Hz		50nM Vitamin D3+1000Hz vs 1000Hz	
	Adherent	Suspension	Adherent	Suspension	Adherent	Suspension
CD14	↑ 559.5 Fold (p = 0.003)	↑ 564.9 Fold (p = 0.016)	No difference (p = 0.24)	No difference (p = 0.41)	↑ 466.0 Fold (p = 0.001)	↑ 422.2 Fold (p = 0.036)
CD36	↑ 9.28 Fold (p = 0.004)	↑ 2.55 Fold (p = 0.0003)	No difference (p = 0.14)	↑ 1.38 Fold (p = 0.008)	↑ 6.73 Fold (p = 0.006)	↑ 1.85 Fold (p = 0.002)
HLA-DRA	No difference (p = 0.57)	↓ 0.15 Fold (p = 0.0001)	No difference (p = 0.87)	No difference (p = 0.14)	No difference (p = 0.39)	↓ 0.18 Fold (p < 0.0001)
HLA-DMB	No difference (p = 0.58)	↑ 2.51 Fold (p = 0.0008)	No difference (p = 0.09)	↑ 1.43 Fold (p = 0.006)	No difference (p = 0.61)	↑ 1.75 Fold (p = 0.002)
NFAT2	No difference (p = 0.075)	No difference (p = 0.14)	No difference (p = 0.28)	No difference (p = 0.15)	No difference (p = 0.052)	↓ 0.84 Fold (p = 0.02)
TCF3	↓ 0.13 Fold (p = 0.031)	↓ 0.58 Fold (p = 0.013)	No difference (p = 0.44)	↓ 0.77 Fold (p = 0.045)	↓ 0.15 Fold (p = 0.009)	No difference (p = 0.08)
TCF4	↓ 0.18 Fold (p = 0.032)	↓ 0.54 Fold (p < 0.0001)	No difference (p = 0.28)	No difference (p = 0.13)	↓ 0.14 Fold (p = 0.029)	↓ 0.59 Fold (p < 0.0001)
TCF7L2	No difference (p = 0.32)	↑ 1.84 Fold (p = 0.0034)	No difference (p = 0.63)	No difference (p = 0.29)	No difference (p = 0.46)	↑ 2.09 Fold (p = 0.0047)
LEF-1	n/a	↓ 0.36 Fold (p = 0.0014)	No difference (p = 0.3)	↓ 0.56 Fold (p = 0.011)	n/a	↓ 0.65 Fold (p = 0.0027)
PKD1	No difference (p = 0.2)	No difference (p = 0.06)	No difference (p = 0.15)	No difference (p = 0.91)	No difference (p = 0.54)	No difference (p = 0.055)
PKD2	↑ 4.09 Fold (p = 0.0036)	↑ 2.2 Fold (p < 0.0001)	No difference (p = 0.16)	No difference (p = 0.74)	↑ 3.24 Fold (p = 0.0001)	↑ 2.3 Fold (p = 0.0039)
PIEZO1	↓ 0.11 Fold (p = 0.026)	↓ 0.59 Fold (p = 0.015)	No difference (p = 0.42)	No difference (p = 0.478)	↓ 0.12 Fold (p = 0.027)	No difference (p = 0.052)