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

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Background: In 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, mechanical, and physical stimuli can also influence 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 vibrations, using qPCR for quantification of transcript expression. 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*), antigen presenting molecules (e.g., *HLA-DRA*), transcription factors (e.g., *LEF-1*, *TCF7L2*), and mechanosensors (e.g., *PIEZO1* and *PKD2*). **Results:** The results showed that vitamin D3 induced THP-1 macrophage differentiation, which was characterized 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 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.

Conclusion: The mechanical stimulation with 1000Hz vibrations resulted in upregulation of *PIEZO1* in THP-1 cells, but it did not affect the differentiation process which was investigated in this study. Vitamin D3 induced THP-1 macrophage differentiation and could potentially influence M2 polarization as observed by upregulation of *CD36* and downregulation of *HLA-DRA*. In addition, in THP-1 cells undergoing the combined stimulation, the gene expression patterns were influenced by vitamin D3, which also ablated the effect of the mechanical stimulus on *PIEZO1* upregulation.

1 **Gene expression during THP-1 differentiation is influenced by vitamin D3 and not**
2 **vibrational mechanostimulation**
3

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17 Abstract

18 **Background:** In injury or infection, monocytes migrate into the affected tissues from
19 circulation and differentiate into macrophages which are subsequently involved in the
20 inflammatory responses. Macrophage differentiation and activation have been studied in
21 response to multiple chemokines and cytokines. However, mechanical, and physical
22 stimuli can also influence macrophage differentiation, activation, cytokine production,
23 and phagocytic activity.
24 **Methods:** In this study, the macrophage differentiation from THP-1 monocytes was
25 assessed upon the stimulation with 1,25-dihydroxyvitamin D3 and 1000Hz vibrations,
26 using qPCR for quantification of transcript expression. Vitamin D binds the vitamin D
27 receptor (VDR) and subsequently modulates the expression of a variety of genes in
28 monocytes. The effects of the 1000Hz vibrational stimulation, and the combined
29 treatment of vitamin D3 and 1000Hz vibrations were unknown. The differentiation of
30 macrophages was assessed by looking at transcription of macrophage markers (e.g.,
31 *CD14*, *CD36*), antigen presenting molecules (e.g., *HLA-DRA*), transcription factors (e.g.,
32 *LEF-1*, *TCF7L2*), and mechanosensors (e.g., *PIEZO1* and *PKD2*).
33 **Results:** The results showed that vitamin D3 induced THP-1 macrophage
34 differentiation, which was characterized by upregulation of *CD14* and *CD36*,
35 downregulation of *HLA-DRA*, upregulation of the *PKD2* (*TRPP2*), and an inverse
36 relationship between *TCF7L2* and *LEF-1*, which were upregulated and downregulated
37 respectively. The 1000Hz vibrations were sensed from the cells which upregulated
38 *PIEZO1* and *TCF3*, but they did not induce expression of genes that would indicate
39 macrophage differentiation. The mRNA transcription profile in the cells stimulated with
40 the combined treatment was comparable to that of the cells stimulated by the vitamin

41   only. The 1000Hz vibrations slightly weakened the effect of the vitamin for the regulation
42   of *CD36* and *HLA-DMB* in the suspension cells, but without causing changes in the
43   regulation patterns. The only exception was the upregulation of *TCF3* in the suspension
44   cells, which was influenced by the vibrations. In the adherent cells, the vitamin D3
45   cancelled the upregulating effect of the 1000Hz vibrations and downregulated *TCF3*.
46   The vitamin also cancelled the upregulation of *PIEZO1* gene by the 1000Hz vibrations
47   in the combined treatment.
48   **Conclusion:** The mechanical stimulation with 1000Hz vibrations resulted in
49   upregulation of *PIEZO1* in THP-1 cells, but it did not affect the differentiation process
50   which was investigated in this study. Vitamin D3 induced THP-1 macrophage
51   differentiation and could potentially influence M2 polarization as observed by
52   upregulation of *CD36* and downregulation of *HLA-DRA*. In addition, in THP-1 cells
53   undergoing the combined stimulation, the gene expression patterns were influenced by
54   vitamin D3, which also ablated the effect of the mechanical stimulus on *PIEZO1*
55   upregulation.
56
Introduction
58   Macrophages play important roles in health and disease through phagocytosis of
59   pathogenic microorganisms, by releasing inflammatory mediators, by inducing and
60   maintaining inflammation, and by removing apoptotic cells and repairing tissues
61   (Gordon, 2007, Mosser and Edwards, 2008). Tissue-resident macrophages, derived
62   from the yolk sac at the embryonic stage, are replicated in tissues to maintain cell
63   number, and have different morphology and function depending on the tissue where
64   they reside (Lavin et al., 2015). However, in the case of tissue injury or infection,
65   monocytes derived from bone marrow circulating in peripheral blood, migrate to the
66   affected tissues where they differentiate into macrophages, and are subsequently
67   involved in the inflammatory response (Shi and Pamer, 2011).
68   THP-1 cells are human immortalized monocytes derived from acute monocytic leukemia
69   and have been extensively used to study macrophage differentiation, functions,
70   signaling pathways, and nutrient and drug transport (Chanput, Mes and Wichers, 2014,
71   Bosshart and Heinzelmann, 2016, Nurminen, Seuter and Carlberg, 2019). In this study
72   we investigated the THP-1 responses towards stimulation with vitamin D3 (1,25-
73   dihydroxyvitamin D3), 1000Hz nanovibrations or both, in order to study the expression
74   of genes that could indicate differentiation or mechanosensitivity changes in these cells.
75   In the following text, the combined treatment refers to the combination of 50 nM vitamin
76   D3 and 1000Hz vibrations. The cell responses were investigated separately in adherent
77   and suspension THP-1 monocytes upon each stimulation, to avoid averaging results for
78   both the cell types within the same population and consider their differences
79   (Supplemental Figure 2).

80   Vitamin D has been shown to promote monocyte differentiation into macrophages and
81   targets multiple genes (Nurminen, Seuter and Carlberg, 2019). The active form of
82   vitamin D, 1,25-dihydroxyvitamin D3, is a lipophilic molecule which easily passes
83   through biological membranes and binds with high-affinity to the receptor and
84   transcription factor vitamin D receptor (VDR), which is primarily located in the nucleus
85   (Haussler et al., 2013). The activation of vitamin D target genes is explained by the
86   chromatin model (Nurminen, Seuter and Carlberg, 2019). The ligand-activated VDR
87   molecules bind to a wide variety of enhancer regions that carry suitable binding motifs
88   and are located within accessible chromatin. With the help of pioneer factors, such as
89   PU.1, CEBPA, and GABPA, VDR increases the accessibility of chromatin at and around
90   these enhancer regions (Seuter, Neme and Carlberg, 2017, 2018, Nurminen et al.,
91   2019). In THP-1 cells, 1,25-dihydroxyvitamin D3 stimulation significantly affects the
92   binding strength of transcription factor CTCF to topologically associating domain (TAD)
93   anchors, which results in about 600 TADs becoming sensitive to vitamin D (Neme,
94   Seuter and Carlberg, 2016). Looping of activated DNA-bound VDR to a transcription
95   start site (TSS) at these promoter regions results in increased chromatin accessibility as
96   well as of H3K27ac and H3K4me3 marks (Seuter, Neme and Carlberg, 2016, Nurminen
97   et al., 2019). All these vitamin D-triggered changes in the local chromatin structure at
98   enhancer and promoter regions finally lead to the activation of RNA polymerase II
99   assembled on the respective TSSs and the start of mRNA synthesis. The vitamin may
100 also affect gene expression by increasing the expression and the activity of transcription
101 factors other than VDR, such as BCL6, NFE2, POU4F2, and ELF4 (Nurminen et al.,
102 2015).
103 The effects of vitamin D have been studied in the context of macrophage differentiation
104 from monocytes, but they are also extended into the effector macrophage responses
105 (Hewison, 2010). In fact, normal human macrophages are able to synthesize 1,25-
106 dihydroxyvitamin D3 when stimulated with interferon gamma (IFNγ) (Phillip Koeffler et
107 al., 1985). The vitamin D is involved in the regulation of T cell and macrophage effector
108 functions, primarily via localized autocrine or paracrine synthesis of 1,25-
109 dihydroxyvitamin D3 from its precursor 25-hydroxyvitamin D_3 (Hewison, 2010). In
110 addition, vitamin D deficiency is prevalent in multiple autoimmune diseases, such as
111 multiple sclerosis, type 1 diabetes, systemic lupus erythematosus, and alopecia areata,
112 and it is highly associated with the risk of autoimmunity (Yang et al., 2013, Lin, Meng
113 and Song, 2019). Vitamin D has been implicated in prevention and protection from
114 autoimmune diseases by immunomodulation of macrophage, dendritic cell, and T cell
115 responses (Hewison, 2010, Yang et al., 2013).
116 In the recent years, interest has been given to the mechanobiology of macrophages,
117 which like other immune cells have evolved mechanisms to perceive and respond to the
118 mechanical forces around them (Kim et al., 2019). The cellular functions of tissue-
119 resident macrophages and monocyte-derived macrophages are affected by the tissue-

120 specific microenvironment, which can create many types of mechanical stress on cells
121 (McWhorter, Davis and Liu, 2015, Mennens, van den Dries and Cambi, 2017). Stiffness
122 and topography, which are mechanical properties of the extracellular matrix, regulate
123 the differentiation, proliferation, and function of macrophages such as phagocytosis
124 (Patel et al., 2012). In monocytes, the PIEZO1 mechanotransduction in response to
125 cyclical hydrostatic pressure, results in HIF1α stabilization and secretion of molecules,
126 such as endothelin-1 (EDN1), and neutrophil chemoattractant CXCL2 (Solis et al.,
127 2019). In addition, macrophages in tissues are exposed to alterations of pressure which
128 affect the secretion of cytokines such as IL-6, TNF-α and IL-1β, (Ferrier et al., 2000,
129 Mevoy et al., 2002). Other mechanical forces that these cells experience originate from
130 dynamic mechanical loading, such as continuous and cyclic stretch and compression
131 (McWhorter, Davis and Liu, 2015, Mennens, van den Dries and Cambi, 2017).
132 Just like normal monocytes, THP-1 cells have shown to respond to mechanical
133 stressors. For example, in models of atherosclerosis, biomechanical strain on THP-1
134 cells can induce expression of the class A scavenger receptor, an important lipoprotein
135 receptor in atherogenesis (Yamamoto, Ikeda and Shimada, 2003). In addition, DNA
136 microarray analysis has shown that cyclic mechanical strain in THP-1 cells induces
137 expression of genes, some encoding for inflammatory markers such as IL-8 and IEX-
138 1(Yamamoto, Ikeda and Shimada, 2003). In these cells, biomechanical deformation
139 influences the degradation of extracellular matrix, monocyte differentiation, and
140 promotion of atherosclerosis (Yamamoto, Ikeda and Shimada, 2003). In addition, as
141 THP-1 cells differentiate they become adherent, a process which may result in altered
142 mechanosensitivity (Tsuchiya et al., 1982, Schwende et al., 1996).
143 In this study, the cells were mechanically stimulated using 1000Hz vibrations. The
144 vibrational stimulation of 1000Hz frequency and nano-scale amplitude has been used to
145 study *in vitro* osteogenic differentiation with successful results (Nikukar et al., 2013,
146 Pemberton et al., 2015, Tsimbouri, 2015, Robertson et al., 2018), and in this study it
147 was used to investigate any effect it may have on the differentiation of macrophages
148 from THP-1 monocytes. Assessment of macrophage differentiation in response to
149 externally applied vibrational stimuli can provide insights into monocyte
150 mechanosensitivity and enquire the therapeutic effects of vibrational treatments in
151 inflammatory diseases.
152 From a technical point of view, the experiments of this study were designed to give an
153 insight into the differentiation process of THP-1 monocytes into macrophages under
154 different stimulation parameters, compare between treatments, and look into
155 mechanosensor mRNA expression.
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Materials & Methods

THP-1 monocyte growth

THP-1 cells (ATCC® TIB-202^{TM}) 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). No medium or vitamin D3 replacement occurred for this duration of time. 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).

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 was 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 bioreactor's stability and generated vibrations were

200 assessed using laser interferometry every 3 months (diagram in Supplemental Figure
201 1). The platform of the bioreactor was generating vibrations of 1000Hz frequency and
202 amplitude range 30-60 nm at the time of the experiments.

RNA extraction

204 The RNA was extracted separately for the suspension and adherent cells. Cell
205 suspension was slowly removed and added to sterile RNase-free 1.5mL tubes. The
206 cells in suspension were pelleted by centrifugation at 3000 rpm for 5 minutes. The
207 supernatant was discarded and 1mL Trizol reagent (Invitrogen, AM9738) was added to
208 homogenize the pellet. For the adherent cells, 1mL Trizol reagent was added directly in
209 the wells. The lysed cells were homogenized using a 25g syringe. The RNA extraction
210 from the lysed cells in Trizol solution was done by separating the aqueous phase after
211 addition of 0.2mL chloroform and centrifugation at 13000 rpm for 15 minutes at 4°C.
212 The RNA was washed with isopropanol and 75% ethanol and stored in 30µL of
213 nuclease-free water (Gibco, 10977035). Quantification of the RNA in ng/µL was done on
214 Nanodrop 1000, using the RNA nucleic acid program.

DNase treatment

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

Complementary DNA synthesis

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

Real-time PCR

230 Real-time PCR was used to quantify gene expression in adherent and suspension THP-
231 1 cells. The PCR amplifications were performed in 25µL reactions containing 12.5µL
232 PowerUP SYBR Green Mastermix (Applied Biosystems™, A25742); 0.5µL Forward
233 Primer and 0.5µL Reverse Primer for the respective genes, 1µL of cDNA and topped up
234 to 25µL with nuclease free water (Gibco, 10977035).
235 The primer pair used for amplification of the housekeeper *RPL37A* were *RPL37A*
236 forward 5'-ATTGAAATCAGCCAGCACGC-3' and *RPL37A* reverse 5'-
237 AGGAACCACAGTGCCAGATCC-3'. The primer pair used for amplification of the
238 housekeeper *ACTB* were *ACTB* forward 5'-ATTGCCGACAGGATGCAGAA-3' and
239 *ACTB* reverse 5'-GCTGATCCACATCTGCTGGAA-3'. The primer pair used for

240 amplification of *CD36* were *CD36* forward 5'-TCACTGCGACATGATTAATGGTACA-3'
241 and *CD36* reverse 5'-ACGTCGGATTCAAATACAGCATAGAT-3'. The primer pair used
242 for amplification of *CD14* were *CD14* forward 5'-ACGCCAGAACCTTGTGAGC-3' and
243 *CD14* reverse 5'-GCATGGATCTCCACCTCTACTG-3'. The primer pair for amplification
244 of *HLA-DRA* were *HLA-DRA* forward 5'-TAAGGCACATGGAGGTGATG-3' and *HLA-*
245 *DRA* reverse 5'-GTACGGAGCAATCGAAGAGG-3'. The primer pair used for
246 amplification of *HLA-DMB* were *HLA-DMB* forward 5'-CTCTCACAGCACCTCAACCA-3'
247 and *HLA-DMB* reverse 5'-TAGAAGCCCCACACATAGCA-3'. The primer pair used for
248 amplification of *PIEZO1* were *PIEZO1* forward 5'-CATCTTGGTGGTCTCCTCTGTCT-3'
249 and *PIEZO1* reverse 5'-CTGGCATCCACATCCCTCTCATC-3'. The primer pair used for
250 detection of *PKD1* were *PKD1* forward 5'-CGCCGCTTCACTAGCTTCGAC-3' and
251 *PKD1* reverse 5'-ACGCTCCAGAGGGAGTCCAC-3'. The primer pair used for
252 amplification of *PKD2* were *PKD2* forward 5'-GCGAGGTCTCTGGGGAAC-3' and *PKD2*
253 reverse 5'-TACACATGGAGCTCATCATGC-3'. The primer pair used for amplification of
254 *NFAT2* were *NFAT2* forward 5'-CACTCCTGCTGCCTTACACA-3' and *NFAT2* reverse
255 5'-AAGATGCGAGCATGCGACTA-3'. The primer pair used for amplification of *TCF3*
256 were *TCF3* forward 5'-TGACCTCCTGGACTTCAGC-3' and *TCF3* reverse 5'-
257 ACCTGAACCTCCGAACTGC-3'. The primer pair used for amplification of *TCF4* were
258 *TCF4* forward 5'-AGTGCGATGTTTTCACCTCC-3' and *TCF4* reverse 5'-
259 CCTGAGCTACTTCTGTCTTC-3'. The primer pair used for the amplification of *TCF7L2*
260 were *TCF7L2* forward 5'-CCGGGAAAGTTTGGAAGAAG-3' and *TCF7L2* reverse 5'-
261 ACTGAAAATGGAGGGTTCGG-3'. The primer pair used for amplification of *LEF-1* were
262 *LEF-1* forward 5'-GACAGTGACCTAATGCACGT-3' and *LEF-1* reverse 5'-
263 CCACCTTCTGCCAAGAATCT-3'.
264 The primers for TCFs and LEF-1 transcription factors were designed and tested by Dr.
265 Robin Freeburn. Primers amplifying *PIEZO1* were designed using the NCBI primer
266 design tool for the mRNA sequence NM_001142864.4, and primers amplifying *CD14*
267 were designed similarly for the mRNA sequences NM_001174105.2 (CD14 mRNA
268 transcript variant 4), NM_001040021.3 (CD14 mRNA transcript variant 2),
269 NM_000591.4 (CD14 mRNA transcript variant 1) and NM_001174104.1 (CD14 mRNA
270 transcript variant 3). The primers for *NFAT2* were obtained from Dagna *et al*. (Dagna,
271 Pritchett and Lusso, 2013), primers for *HLA-DRA* and *HLA-DMB* were obtained from
272 Ulbricht *et al*. (Ulbricht et al., 2012), primers for *PKD1* and *PKD2* were obtained from
273 Dalagiorgou *et al*. (Dalagiorgou et al., 2013), and primers from *CD36*, *ACTB* and
274 *RPL37A* were obtained from Maeß *et al.* (Maeß, Sendelbach and Lorkowski, 2010).
275 The efficiency of primers taken from existing literature has been assessed in published
276 papers (Fukuda, Mitsuoka and Schmid-Schönbein, 2004, Maeß, Sendelbach and
277 Lorkowski, 2010, Ulbricht et al., 2012, Dagna, Pritchett and Lusso, 2013, Dalagiorgou et
278 al., 2013). The primer efficiency was assessed prior to the experiments and was around
279 97% for all the investigated genes. Similar PCR efficiency for each primer is necessary

280   for relative quantification using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). The
281   PCR efficiency was also assessed by melt curve analysis. The collected C_T values were
282   used for the $\Delta\Delta C_T$ relative quantification of expression, comparing the stimulated cells
283   to the untreated controls. The ΔC_T was obtained by comparison of CTs of genes of
284   interest to the mean C_T of two housekeeping genes *RPL37A* and *ACTB.* These
285   housekeeping genes are considered to be the best for the analysis of RNA expression
286   in THP-1 cells (Maeß, Sendelbach and Lorkowski, 2010).

287   **Statistical analysis**

288   The gene expression data are presented as mean of four replicates ± SEM, with little
289   exception where some particular genes were not detected in all replicates. The analysis
290   of statistical significance between the stimulated cells versus controls, and between
291   each type of stimulation was done using unpaired T test with Welch's correction.
292   Statistical analysis was carried out using GraphPad Prism® version 6. P values <0.05
293   were accepted as significant.

294   **Results**

295   **Regulation of genes encoding macrophage markers and antigen presenting**
296   **molecules**

297

298   Stimulation with vitamin D3, which also served as a positive control for the induction of
299   differentiation, resulted in upregulation of the *CD14* and *CD36* mRNA in both adherent
300   and suspension cells (Figure 1, A; Figure 2, A). Vitamin D3 also downregulated the
301   *HLA-DRA* expression in adherent and suspension cells (Figure 1, A; Figure 2, A). The
302   mRNA of *HLA-DMB* was upregulated for vitamin D3 stimulation in suspension cells
303   (Figure 1, A). The *HLA-DMB* was not regulated in response to the vitamin D3 in the
304   adherent cells (Figure 2, A).
305   The 1000Hz stimulation caused upregulation of *CD36* and downregulation of *HLA-DMB*
306   in suspension cells (Figure 1, B), whereas in adherent cells it only downregulated the
307   *HLA-DRA* (Figure 2, B).
308   The combined stimulation induced upregulation of *CD14* and *CD36* in both adherent
309   and suspension cells (Figure 1, C; Figure 2, C). The *HLA-DRA* was downregulated in
310   both cell types compared to the respective unstimulated control (Figure 1, C; Figure 2,
311   C), whereas *HLA-DMB* was upregulated in suspension cells (Figure 1, C).
312   The expression values of *CD14, CD36, HLA-DRA* and *HLA-DMB* in stimulated cells
313   versus controls are shown in Table 1 and Table 2, for suspension and adherent cells
314   respectively.
315   A comparison between the treatments was performed for the above genes in
316   suspension (Figure 3) and adherent cells (Figure 4). The comparison between
317   treatments is shown in detail in Table 3.
318   *CD14* was upregulated only in response to the vitamin D3, as the mRNA levels were
319   comparable to the cells stimulated by the vitamin only (Figure 3, A; Figure 4, A).

320   Similarly, the upregulation of *CD36* in the adherent cells was only in response to the
321   vitamin D3 in the combined treatment (Figure 3, A). In suspension cells undergoing the
322   combined treatment, the 1000Hz stimulation weakened the upregulation of *CD36* by the
323   vitamin D3, which was still higher than the upregulation caused by the 1000Hz
324   vibrational stimulation alone (Table 3). In the combined treatment, the 1000Hz
325   vibrations also weakened the upregulation of *HLA-DMB* by the vitamin in the
326   suspension cells (Table 3). Interestingly, the *HLA-DRA* was downregulated from all
327   treatments at the same level in the adherent cells (Figure 4, A), but only the vitamin
328   downregulated this gene in suspension cells (Figure 3, A; Table 3).
329
330   **Regulation of genes encoding transcription factor**
331
332   The stimulation with vitamin D3 downregulated *NFAT2* and *TCF3* in adherent cells
333   (Figure 2, A). The *TCF4* and *LEF-1* were downregulated in both adherent and
334   suspension cells stimulated with the vitamin (Figure 1, A; Figure 2, A). The *TCF7L2*
335   mRNA was upregulated in response to the stimulation with vitamin D3 in suspension
336   (Figure 1, A), and adherent cells (Figure 2, A).
337   The 1000Hz vibrational stimulation upregulated *TCF3* in both adherent and suspension
338   cells compared to the respective controls (Figure 1, B; Figure 2, B). This type of
339   stimulation also downregulated *NFAT2* in the adherent cells (Figure 2, B). The mRNA
340   expression of *TCF4*, *TCF7L2* and *LEF-1* were not affected by the vibrational stimulation
341   (Figure 1, B; Figure 2, B).
342   The combined stimulation downregulated *NFAT2* in both adherent and suspension cells
343   (Figure 1, C; Figure 2, C). The *TCF3* mRNA was downregulated in the adherent cells
344   (Figure 2, C), but upregulated in the suspension cells (Figure 1, C). The *TCF4* was
345   downregulated in both cell types, and *TCF7L2* was upregulated in both cell types
346   (Figure 1, C; Figure 2, C). The *LEF-1* was downregulated in the suspension cells
347   (Figure 1, C).
348   The expression values of *TCF3, TCF4, TCF7L2* and *LEF-1* in stimulated cells versus
349   controls are also shown in Table 1 and Table 2, for suspension and adherent cells
350   respectively.
351   A comparison between the treatments was performed for these genes encoding
352   transcription factors in suspension (Figure 3, B) and adherent cells (Figure 4, B), and
353   shown in Table 3.
354   The *NFAT2* mRNA was downregulated in adherent cells for all the treatments, without
355   difference between each other (Figure 4, B). In the suspension cells, the *NFAT2* was
356   downregulated only for the combined stimulation (Figure 3, B).
357   In adherent cells, the *TCF3* mRNA was downregulated in response to vitamin D3 but
358   upregulated for the 1000Hz stimulation. In the adherent cells, the vitamin cancelled the
359   upregulating effect of the 1000Hz vibration and downregulated *TCF3*, at comparable

360 levels to the cells stimulated with vitamin D3 only (Figure 4, B; Table 3). However, in
361 suspension cells, the *TCF3* upregulation was influenced by the 1000Hz vibrations, and
362 the mRNA levels were comparable to the cells stimulated with the 1000Hz vibrations
363 alone (Table 3; Figure 3, B).
364 The *TCF4* mRNA was downregulated in response to vitamin D3 stimulation in both
365 suspension and adherent cells. In the combined treatment, *TCF4* was influenced by the
366 vitamin only. The 1000Hz did not have any influence on the expression of this gene
367 neither alone nor in combination with the vitamin (Figure 3, B; Figure 4, B). Similarly, the
368 upregulation of *TCF7L2* mRNA was influenced only by the vitamin D3 in both adherent
369 and suspension cells, with the 1000Hz stimulation having no effect on the cells when
370 applied alone or in combination with the vitamin (Figure 3, B; Figure 4, B).
371 The mRNA for *LEF-1* was downregulated in response to vitamin D3 stimulation. In the
372 adherent cells little RNA was obtained for this gene, and no amplification was detected
373 for the 1000Hz stimulation (Table 3). This needs to be investigated in the future to
374 explain whether the lack of amplification was due to very low transcripts levels in total
375 RNA, or because of some inhibitory effect that 1000Hz vibrations may have. In the
376 suspension cells stimulated with the combined treatment, the 1000Hz weakened the
377 downregulating effect of the vitamin D3, however the vitamin influenced the
378 downregulation (Figure 3, B).
379
380 **Regulation of genes encoding mechanosensors *PIEZO1, PKD1* and *PKD2***
381
382 The stimulation with the vitamin D3 resulted in upregulation of *PKD2* (*TRPP2*) mRNA in
383 both adherent and suspension cells. The stimulation with vitamin D3 alone did not affect
384 the expression of *PIEZO1* or *PKD1* (*TRPP1*) (Figure 1, A; Figure 2, A).
385 The 1000Hz vibrational stimulation resulted in upregulation of *PIEZO1* mRNA in both
386 adherent and suspension cells. In the adherent cells, the stimulation downregulated
387 *PKD2* mRNA. The vibrational stimulation did not affect *PKD1* expression (Figure 1, B
388 Figure 2, B).
389 The combined treatment resulted in the upregulation of *PKD2* mRNA in both adherent
390 and suspension cells. *PIEZO1* and *PKD1* were not regulated in cells stimulated with the
391 combined treatment (Figure 1, C Figure 2, C).
392 The expression patterns of *PKD2* and *PIEZO1* in stimulated cells versus controls are
393 shown in Table 1 and Table 2, for suspension and adherent cells, respectively.
394 A comparison between treatments was performed for these genes encoding
395 mechanosensors in the suspension (Figure 3, C) and adherent cells (Figure 4, C), and
396 shown in Table 3.
397 The expression of *PKD2* was affected only by the vitamin D3, which also cancelled the
398 downregulation effect of the 1000Hz in the adherent cells stimulated with the combined
399 treatment (Table 3).

400 The *PIEZO1* upregulation occurred only in response to the stimulation with 1000Hz
401 vibrations, but in the combined treatment the vitamin cancelled the upregulating effect of
402 the vibrational stimulation (Table 3). The expression of *PKD1* mRNA was not affected
403 by any of the stimulation methods (Table 3).
404 A comparison of *PIEZO1* expression between adherent and suspension cells stimulated
405 with 1000Hz vibrations and the combined treatment was performed (Supplemental
406 Figure 3). The 1000Hz vibrations upregulated *PIEZO1* stronger in adherent cells, than
407 in the suspension cells (Supplemental Figure 3, A). No difference was observed
408 between the adherent and suspension cells stimulated with the combined treatment
409 (Supplemental Figure 3, B).
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414 **Discussion**
415
416 **TCF/LEF pathway and gene nomenclature**
417
418 TCF/LEF pathway plays roles in monocyte and macrophage differentiation (Thiele et al.,
419 2001). It must be mentioned that some confusion exists about the nomenclature of the
420 TCFs. The mammalian TCF/LEF family comprises of four nuclear factors designated
421 TCF7, LEF1, TCF7L1, and TCF7L2, which are also known as TCF1, LEF1, TCF3, and
422 TCF4, respectively (Hrckulak et al., 2016). Confusion also exists between the
423 nomenclature of genes and the corresponding products. For example, a gene called
424 *TCF3* (NCBI gene ID: 6929), also known as *E2A*, encodes a product that is different
425 from TCF3 encoded from *TCF7L1* (NCBI Gene ID: 83439). Similarly, *TCF4* (NCBI gene
426 ID: 6925), encodes for TCF4 which is a different protein from the TCF4 encoded from
427 *TCF7L2* (NCBI Gene ID: 6934). In this experiment, the mRNA investigated belongs to
428 genes *TCF3* (E2A), *TCF4* (E2-2), *TCF7L2* and *LEF-1*, with the last two investigated in
429 the context of WNT canonical pathways in monocyte-derived macrophages (Malsin et
430 al., 2019). The pathways which involve *TCF3* and *TCF4* gene products can be complex
431 and are not elucidated in context of monocyte to macrophage differentiation.
432
433 **Vitamin D3 induced macrophage differentiation and downregulated *HLA-DRA***
434
435 Vitamin D3 has shown to target multiple monocyte genes and promote monocyte
436 differentiation into macrophages (Nurminen, Seuter and Carlberg, 2019). Similarly, this
437 study demonstrated that vitamin D3 stimulation induced differentiation of THP-1
438 monocytes into macrophages, when looking at transcriptional regulation of *CD14*, *CD36*
439 and transcription factors *TCF7L2* (encoding TCF4) and *LEF-1* (Table, 1; Table 2).

440 The stimulation with vitamin D3 resulted in upregulation of the *CD14* and *CD36* mRNA
441 in both adherent and suspension cells. This pattern of regulation for these two genes
442 was expected to occur during macrophage differentiation from monocytes (Zhang et al.,
443 1994, Maeß, Sendelbach and Lorkowski, 2010). The CD14 is an important marker of
444 the THP-1 differentiation into macrophages which upregulates strongly upon vitamin D3
445 stimulation (Schwende et al., 1996, Gocek et al., 2012), as was also observed in this
446 study. Furthermore, *CD14* and *CD36* are primary target genes for vitamin D3 in THP-1
447 monocytes (Nurminen, Seuter and Carlberg, 2019). The CD14 and CD36 are proteins
448 involved in macrophage functions. CD14 cooperates with Toll-like receptor 4 (TLR4) to
449 mediate the macrophage immune response to bacterial lipopolysaccharide
450 (LPS)(Zanoni et al., 2011).
451 CD36 is a scavenger receptor which has been associated with M2 polarization and
452 enhanced phagocytosis (Pennathur et al., 2015, Woo et al., 2016).
453 It has been reported that M2 activation of bone marrow-derived macrophages with IL-4
454 has resulted in upregulation of CD36 expression, whereas M1 activation with LPS and
455 interferon-γ has resulted in downregulation of the receptor (Pennathur et al., 2015).
456 During kidney injury, CD36 is an important phenotypic marker of profibrotic M2
457 macrophages and a key phagocytic receptor for the clearance of apoptotic cells
458 (Pennathur et al., 2015). Similarly, during the resolution phase of stroke, CD36
459 macrophages have a reparative role through phagocytosis (Woo et al., 2016).
460 Vitamin D3 downregulated the expression of *HLA-DRA* in differentiating THP-1 cells
461 (Table, 1; Table 2). HLA-DR has been described as an M1 marker, which is upregulated
462 in THP-1 and monocyte - derived macrophages stimulated with IFNγ/LPS, whereas its
463 expression is very low with IL-4/IL-13 stimulation (Yang et al., 2016). The decreased
464 HLA-DR expression in monocytes has also been associated with anti-inflammatory
465 states or immunosuppression. HLA-DR expression is decreased in all monocyte
466 subsets upon IL-10 exposure *in vitro* and during septic shock (Monneret et al., 2004,
467 Lee et al., 2017), whereas monocytes that have diminished or no HLA-DR expression,
468 called CD14+HLA-DR[lo/neg] monocytes, have emerged as important mediators of tumor-
469 induced immunosuppression (Mengos, Gastineau and Gustafson, 2019).
470 Downregulation of the HLA-DR protein has been observed in primary monocytes
471 treated with vitamin D3 (Tokuda and Levy, 1996), as well as in dendritic cells (Ferreira
472 et al., 2015). In dendritic cells, the downregulation of HLA-DR has been suggested to be
473 part of tolerance processes induced by vitamin D3 signaling (Ferreira et al., 2015).
474 The upregulation of *CD36* and downregulation of *HLA-DRA* mRNA by vitamin D3 in day
475 3-differentiating THP-1 macrophages, could indicate predisposition for M2 polarization.
476 In addition, vitamin D3 stimulation upregulated the mRNA of *HLA-DMB* in suspension
477 cells. This molecule is important for antigen loading of the MHC class II by removal of
478 CLIP from HLA-DR (Riberdy et al., 1992, Sloan et al., 1995). In one study, HIV-infected
479 THP-1 monocytes had loss of mRNA for *HLA-DR*, but the mRNAs for *HLA-DM*

480 continued to be transcribed, showing that genes may have non-corresponding
481 expression patterns (Shao and Sperber, 2002), similar to what was observed in this
482 study.
483 This study also identified an inverse relationship between *TCF7L2* and *LEF-1* mRNA
484 regulation during vitamin D3-induced macrophage differentiation. The *TCF7L2*
485 (encoding TCF4) in combination with β-catenin forms a complex that regulates
486 expression of genes in monocytes and it is thus involved in the differentiation process
487 (Thiele et al., 2001, Tickenbrock, 2006, Malsin et al., 2019), whereas LEF-1 facilitates
488 nuclear localization of β-catenin and enhances proliferation in acute myeloid leukemia
489 cells, including THP-1 cells (Morgan et al., 2019). Therefore, the downregulation of *LEF-*
490 *1* and the upregulation of *TCF7L2* could indicate decreased proliferation and increased
491 differentiation as THP-1 monocytes become macrophages (Schwende et al., 1996,
492 Thiele et al., 2001, Morgan et al., 2019). The inverse relationship of *TCF7L2* and *LEF-1*
493 has also been related to shifts in differentiation and proliferation states in other cancer
494 cells (Kriegl et al., 2010, Eichhoff et al., 2011). This pattern of regulation for these two
495 genes can be signature of THP-1 monocyte to macrophage differentiation.
496 Another transcription factor downregulated in adherent cells in response to the vitamin
497 D3 was *NFAT2*. The NFATs are important transcription factors for production of
498 proinflammatory cytokines in T and B cells (Macian, 2005), but their roles are not only
499 limited to the adaptive immune cells. It has been showed that the NFATs are required
500 for Toll-like receptor (TLR)-initiated innate immune responses in bone marrow-derived
501 macrophages (Minematsu et al., 2011). In THP-1 monocytes *in vitro*, the NFAT2 has
502 shown to inhibit the release of high mobility protein box-1 (HMGB1) (Zhao Q et al.,
503 2016), a proinflammatory protein with roles in inflammation and autoimmunity (Magna
504 and Pisetsky, 2014). The suppression of NFAT2 expression by siRNA has resulted in
505 increased HMGB1 in the supernatant of cells (Zhao Q et al., 2016). In T cells, 1,25-
506 dihydroxyvitamin D3 and its receptor complex (VDR-RXR) have shown to inhibit NFAT
507 activity (Wöbke, Sorg and Steinhilber, 2014), but its effect on monocytes and *NFAT2*
508 mRNA are not known. In this study, the downregulation of *NFAT2* mRNA in the
509 adherent cells, which are considered to be in a more advanced stage of differentiation
510 than the suspension cells (Tsuchiya et al., 1982, Schwende et al., 1996)(Supplemental
511 Figure 2), could be related to the production of proinflammatory proteins after the
512 maturation of the monocytes into macrophages.
513 The vitamin D3 stimulation also downregulated *TCF3* (encoding E2A) in adherent cells,
514 and *TCF4* (encoding E2-2) in both cell types compared to the respective controls. The
515 roles of the products of these genes are not known in monocyte biology and
516 macrophage differentiation, but as demonstrated in this study they are regulatable upon
517 vitamin D3 stimulation.
518 The stimulation with vitamin D3 had no effect on the regulation of *PIEZO1* or *PKD1*, but
519 it upregulated *PKD2* (*TRPP2*) mRNA in both suspension and adhesion cells. The roles

520 of polycystin 2 (product of *PKD2*) are not known in THP-1 monocytes, but the results of
521 this study suggest that the *PKD2* mRNA upregulation can be signature of vitamin D3-
522 induced differentiation.
523 **Monocyte responses to 1000Hz vibrational stimulation**
524
525 The THP-1 monocytes are responsive to mechanical stressors. Biomechanical strain on
526 THP-1 cells can induce expression of the class A scavenger receptor, degradation of
527 extracellular matrix, monocyte differentiation, and promotion of atherosclerosis
528 (Yamamoto, Ikeda and Shimada, 2003). In addition, DNA microarray analysis has
529 shown that cyclic mechanical strain in THP-1 cells induces expression of genes, some
530 encoding for inflammatory markers such as IL-8 and IEX-1 (Yamamoto, Ikeda and
531 Shimada, 2003). Furthermore, upon differentiation, THP-1 cells become adherent
532 (Tsuchiya et al., 1982, Schwende et al., 1996), which may result in altered
533 mechanosensitivity. This study used 1000Hz vibrations as artificially applied mechanical
534 stimulation, in order to study the mechanosensitivity of THP-1 monocytes and assess if
535 it could affect macrophage differentiation.
536 The vibrational 1000Hz stimulation resulted in upregulation of *PIEZO1* transcripts in
537 both suspension (Table 1) and adhesion cells (Table 2). PIEZO1 channels are
538 considered professional mechanosensory proteins, capable of sensing and converting
539 mechanical stimuli (Zhong et al., 2018). Little is known about the mechanosensory roles
540 of these channels in monocytes and macrophages. RNA expression analysis presented
541 in cell atlas shows *PIEZO1* expressed in monocytes and macrophages, as well as in
542 THP-1 cells (Human Protein Atlas, Cell Type RNA, Piezo1). *PIEZO2* expression has not
543 been detected in blood cells, including monocytes, whereas its expression in THP-1
544 cells is negligible (Human Protein Atlas, Cell Type RNA, Piezo2).
545 In monocytes, PIEZO1 has shown to signal in response to cyclical hydrostatic pressure,
546 resulting in HIF1α stabilization and secretion of molecules, such as endothelin-1
547 (EDN1), and neutrophil chemoattractant CXCL2 (Solis et al., 2019). The PIEZO1
548 signaling to the cyclical pressure has induced inflammation and infiltration of
549 monocytes, which recruit neutrophils in order to clear pulmonary *Pseudomonas*
550 *aeruginosa* infection via EDN1 (Solis et al., 2019). In this study, we demonstrated that
551 THP-1 cells upregulate *PIEZO1* mRNA in response to 1000Hz vibrational stimulation,
552 when applied in isolation. However, the biological significance of such regulation
553 remains to be elucidated. In addition, the *PIEZO1* mRNA upregulation in response to
554 the 1000Hz vibrations was stronger in the adherent cells that were in contact with the
555 vibrating surface, compared to the floating suspension cells (Supplemental Figure 3, A).
556 This could indicate potential involvement of mechanotransduction for the regulation
557 of *PIEZO1* expression in 1000Hz vibrated THP-1 monocytes.
558 The 1000Hz stimulation also caused *HLA-DRA* downregulation in adherent cells like
559 vitamin D3, but when combined with the vitamin it did not show any synergetic effect

560   (Table 3). Another gene which was upregulated during the stimulation with 1000Hz
561   vibrations, was *TCF3*. This gene was upregulated in both suspension and adherent
562   cells (Table 1; Table 2), but the role of this gene and its products are not known in
563   monocytes. The 1000Hz vibrations downregulated the *NFAT2* mRNA at the same levels
564   as the vitamin D3 in adherent cells (Table 2), and just like the vitamin it did not regulate
565   this gene in suspension cells (Table 1).
566   The vibrational stimulation had no effect on the regulation of other transcription factors
567   such as *TCF4*, *TCF7L2* and *LEF-1,* which were influenced by the vitamin D3 only (Table
568   1; Table 2; Table 3).
569   The upregulation of *PIEZO1* and *TCF3* upon the application of the 1000Hz stimulation
570   was interesting, but it was not associated with macrophage differentiation, because
571   there was no transcriptional regulation for genes such as *CD14*, *TCF7L2* and *LEF-1*
572   which would indicate transition from monocytes to macrophages. The *CD36* was
573   upregulated for the 1000Hz stimulation in suspension cells. However, in adherent cells,
574   the *CD36* mRNA levels were comparable to the unstimulated adherent controls.
575
576   **The effects of the combined treatment on gene expression and comparison to**
577   **vitamin D3 and 1000Hz vibrations**
578
579   The combined treatment induced macrophages differentiation, but the process was
580   influenced mostly by the vitamin D3 (Table 3).
581   The upregulation of *CD14* in suspension and adherent cells undergoing the combined
582   treatment was comparable to cells stimulated with vitamin D3 only (Table 3). The *CD36*
583   mRNA was upregulated in the adherent cells at comparable level to the cells stimulated
584   with vitamin D3 only. However, in the suspension cells the 1000Hz had slightly
585   weakened the upregulation of *CD36* by the vitamin D3. The combination of both stimuli
586   resulted in lower mRNA expression than the stimulation with the vitamin, but higher than
587   the stimulation with the 1000Hz, hence it could be said that the 1000Hz weakened the
588   upregulating effect of the vitamin (Table 3).
589   Even though when applied in isolation the 1000Hz stimulation caused *HLA-DRA*
590   downregulation in adherent cells at similar levels to vitamin D3, in the combined
591   stimulation it did not show any synergetic effect. The downregulation of *HLA-DRA* in
592   suspension cells undergoing the combined treatment was comparable to the cells
593   stimulated with the vitamin D3 only, showing that in the combined treatment this gene
594   was influenced only by the vitamin (Table 3).
595   In the suspension cells undergoing the combined treatment, the 1000Hz weakened the
596   upregulation of *HLA-DMB* by the vitamin. When applied in isolation the 1000Hz
597   vibrations downregulated *HLA-DMB*, however, in the combined treatment the vitamin
598   overshadowed the effect of the vibrational stimulus and caused upregulation (Table 3).

599   The combined treatment downregulated *NFAT2* at comparable levels to both the
600   vitamin D3 and 1000Hz treatments when applied alone in the adherent cells. However,
601   in the suspension cells, the 1000Hz and the vitamin D3 may have synergistically caused
602   the downregulation of *NFAT2* in suspension cells, because the vitamin and the 1000Hz
603   did not regulate this gene when applied in isolation.
604   The regulation of *TCF4* and *TCF7L2* in the cells stimulated with the combined treatment
605   was comparable to the cells stimulated with vitamin D3, and the 1000Hz stimulation had
606   no effect on these genes in the combined treatment (Table 3), similar to when it was
607   applied in isolation (Table 1; Table 2). The 1000Hz vibrations however, weakened the
608   downregulating effect that the vitamin had on the mRNA encoding *LEF-1* in the
609   suspension cell. In the adherent cells, the *LEF-1* mRNA in stimulated with the combined
610   treatment was comparable to the unstimulated controls, but since the mRNA for this
611   gene was not detected in cell stimulated with vibrations only, comparison could not take
612   place (Table 3).
613   In the presence of the vitamin D3, the effect of 1000Hz stimulation on the regulation of
614   *PIEZO1* was cancelled in both adherent and suspension cells. Furthermore, in adherent
615   cells, the vitamin D3 cancelled the upregulating effect of 1000Hz on the *TCF3* and
616   downregulated the gene (Table 2, Table 3). However, in suspension cells the 1000Hz
617   stimulation continued to upregulate *TCF3* even in the presence of the vitamin (Table 3).
618   This was the only case in which the effects of 1000Hz strongly influenced the
619   expression pattern of a gene in the presence of the vitamin.
620

621   **Conclusions**
622   This study demonstrated that the stimulation with 50nM vitamin D3 for 3 days drives
623   THP-1 macrophage differentiation, as was determined by upregulation of *CD14*, *CD36*
624   and *TCF7L2*, and downregulation of *LEF-1*. The differentiation induced by vitamin D3
625   was accompanied by downregulation of *HLA-DRA* and upregulation of *PKD2* mRNA.
626   Other genes that were regulated during vitamin D3-induced macrophage differentiation
627   included *TCF3* and *TCF4* in both suspension and adherent cells, and *NFAT2* in
628   adherent cells. The upregulation of the mechanosensitive non-selective cation channel
629   *PKD2* mRNA could suggest a role during THP-1 macrophage differentiation, whereas
630   the upregulation of *CD36* and downregulation of *HLA-DRA* mRNA could be indicative of
631   predisposition for M2 polarization.
632   The vibrational stimulation which was used for the mechanical stimulation of cells did
633   not induce the macrophage differentiation process because there was no transcriptional
634   regulation of *CD14* and *TCF/LEF* transcription factors. However, the 1000Hz vibrations
635   influenced upregulation of *PIEZO1* and *TCF3* in both adherent and suspension cells.
636   Furthermore, in adherent cells, the vibrational stimulation downregulated *NFAT2* and
637   *HLA-DRA* at comparable levels to the vitamin D3 stimulated adherent cells. This
638   indicated that while the 1000Hz vibrations did not induce differentiation, they induced

639 regulation of genes in the THP-1 cells. However, the biological importance of such
640 response remains to be elucidated.
641 In the combined treatment, the 1000Hz vibrations interfered with the regulation of some
642 genes by the vitamin D3 but without changing their regulation pattern. The only
643 exception was *TCF3* in suspension cells stimulated with the combined treatment, which
644 was upregulated by the 1000Hz vibrations against the downregulating influence of the
645 vitamin D3. The biological importance of such interference remains to be elucidated.
646 However, the mRNA regulation patterns of the other genes of interest in the combined
647 treatment were in response to vitamin D3 stimulation.
648 Furthermore, the influence of the 1000Hz stimulus in the presence of the vitamin D3
649 was cancelled (e.g. for *PIEZO1* in both cell types), overshadowed (e.g. for *CD36* in
650 suspension cells), or cancelled and reversed (e.g. *PKD2* in adherent cells). This can
651 have implication for the medicinal application of the 1000Hz (nano-scale amplitude)
652 vibrations, because in inflamed tissues rich in chemical signals such as cytokines and
653 chemokines, the cells may lose the ability to sense and respond to such mechanical
654 stimulus.
655 Further work is necessary to assess the reproducibility of the observations of this study,
656 especially in response to the 1000Hz vibrational stimulation. This study was limited by
657 the technology, which was not provided for repeated runs and further work. Increased
658 replicates, expanded time-points, assessment of protein expression, and use of primary
659 monocytes to compare to THP-1 cell responses, are recommended for future work from
660 the authors of this report. In addition, the effects of different frequencies and of
661 vibrations applied in cyclical short-term patterns remain to be studied, in order to
662 expand our understanding of THP-1 cell responses towards the vibrational stimulation.
663 Overall, this study presents experimental results indicating that the vibrational
664 mechanical forces can be sensed by THP-1 monocytes, but that the chemical ligands
665 such as vitamin D3 remain superior for the induction of macrophage differentiation.
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703 **References**
704 Bosshart, H. and Heinzelmann, M. (2016) THP-1 cells as a model for human monocytes. Annals
705   of Translational Medicine. [Online] Vol.4 (21), pp.438–438. Available:
706   http://atm.amegroups.com/article/view/11701/12871.
707 Chanput, W., Mes, J. J. and Wichers, H. J. (2014) THP-1 cell line: An in vitro cell model for
708   immune modulation approach. International Immunopharmacology. [Online] Vol.23 (1),
709   pp.37–45. Available: https://linkinghub.elsevier.com/retrieve/pii/S1567576914003166.
710 Dagna, L., Pritchett, J. C. and Lusso, P. (2013) Immunomodulation and immunosuppression by
711   human herpesvirus 6A and 6B. Future virology. [Online] Vol.8 (3), pp.273–287. Available:
712   http://www.ncbi.nlm.nih.gov/pubmed/24163703.
713 Dalagiorgou, G., Piperi, C., Georgopoulou, U., Adamopoulos, C., Basdra, E. K. and Papavassiliou,
714   A. G. (2013) Mechanical stimulation of polycystin-1 induces human osteoblastic gene
715   expression via potentiation of the calcineurin/NFAT signaling axis. Cellular and Molecular
716   Life Sciences. [Online] Vol.70 (1), pp.167–180. Available:
717   http://link.springer.com/10.1007/s00018-012-1164-5.
718 Eichhoff, O. M., Weeraratna, A., Zipser, M. C., Denat, L., Widmer, D. S., Xu, M., Kriegl, L.,

763 Lee, J., Tam, H., Adler, L., Ilstad-Minnihan, A., Macaubas, C. and Mellins, E. D. (2017) The MHC
764   class II antigen presentation pathway in human monocytes differs by subset and is
765   regulated by cytokines. PLOS ONE. [Online] Vol.12 (8), p.e0183594. Available:
766   https://dx.plos.org/10.1371/journal.pone.0183594.
767 Lin, X., Meng, X. and Song, Z. (2019) Vitamin D and alopecia areata: possible roles in
768   pathogenesis and potential implications for therapy. American journal of translational
769   research. [Online] Vol.11 (9), pp.5285–5300. Available:
770   http://www.ncbi.nlm.nih.gov/pubmed/31632510.
771 Livak, K. J. and Schmittgen, T. D. (2001) Analysis of Relative Gene Expression Data Using Real-
772   Time Quantitative PCR and the 2−ΔΔCT Method. Methods. [Online] Vol.25 (4), pp.402–408.
773   Available: https://linkinghub.elsevier.com/retrieve/pii/S1046202301912629.
774 Macian, F. (2005) NFAT proteins: key regulators of T-cell development and function. Nature
775   Reviews Immunology. [Online] Vol.5 (6), pp.472–484. Available:
776   http://www.nature.com/articles/nri1632.
777 Maeß, M. B., Sendelbach, S. and Lorkowski, S. (2010) Selection of reliable reference genes
778   during THP-1 monocyte differentiation into macrophages. BMC Molecular Biology. [Online]
779   Vol.11 (1), p.90. Available: http://bmcmolbiol.biomedcentral.com/articles/10.1186/1471-
780   2199-11-90.
781 Magna, M. and Pisetsky, D. S. (2014) The Role of HMGB1 in the Pathogenesis of Inflammatory
782   and Autoimmune Diseases. Molecular Medicine. [Online] Vol.20 (1), pp.138–146.
783   Available: https://molmed.biomedcentral.com/articles/10.2119/molmed.2013.00164.
784 Malsin, E. S., Kim, S., Lam, A. P. and Gottardi, C. J. (2019) Macrophages as a Source and
785   Recipient of Wnt Signals. Frontiers in Immunology. [Online] Vol.10. Available:
786   https://www.frontiersin.org/article/10.3389/fimmu.2019.01813/full.
787 McWhorter, F. Y., Davis, C. T. and Liu, W. F. (2015) Physical and mechanical regulation of
788   macrophage phenotype and function. Cellular and Molecular Life Sciences. [Online] Vol.72
789   (7), pp.1303–1316. Available: http://link.springer.com/10.1007/s00018-014-1796-8.
790 Mengos, A. E., Gastineau, D. A. and Gustafson, M. P. (2019) The CD14+HLA-DRlo/neg
791   Monocyte: An Immunosuppressive Phenotype That Restrains Responses to Cancer
792   Immunotherapy. Frontiers in Immunology. [Online] Vol.10. Available:
793   https://www.frontiersin.org/article/10.3389/fimmu.2019.01147/full.
794 Mennens, S. F. B., van den Dries, K. and Cambi, A. (2017) Role for Mechanotransduction in
795   Macrophage and Dendrite Cell Immunobiology. In: Results and Problems in Cell
796   Differentiation. [Online]. pp.209–242. Available: http://link.springer.com/10.1007/978-3-
797   319-54090-0_9.
798 Mevoy, A., Jeyam, M., Ferrier, G., Evans, C. . and Andrew, J. . (2002) Synergistic effect of
799   particles and cyclic pressure on cytokine production in human monocyte/macrophages:
800   proposed role in periprosthetic osteolysis. Bone. [Online] Vol.30 (1), pp.171–177.
801   Available: http://linkinghub.elsevier.com/retrieve/pii/S8756328201006585.
802 Minematsu, H., Shin, M. J., Celil Aydemir, A. B., Kim, K.-O., Nizami, S. A., Chung, G.-J. and Lee, F.
803   Y.-I. (2011) Nuclear presence of nuclear factor of activated T cells (NFAT) c3 and c4 is
804   required for Toll-like receptor-activated innate inflammatory response of
805   monocytes/macrophages. Cellular Signalling. [Online] Vol.23 (11), pp.1785–1793.
806   Available: https://linkinghub.elsevier.com/retrieve/pii/S0898656811001859.

807 Monneret, G., Finck, M.-E., Venet, F., Debard, A.-L., Bohé, J., Bienvenu, J. and Lepape, A. (2004)
808   The anti-inflammatory response dominates after septic shock: association of low
809   monocyte HLA-DR expression and high interleukin-10 concentration. Immunology Letters.
810   [Online] Vol.95 (2), pp.193–198. Available:
811   https://linkinghub.elsevier.com/retrieve/pii/S0165247804001993.
812 Morgan, R. G., Ridsdale, J., Payne, M., Heesom, K. J., Wilson, M. C., Davidson, A., Greenhough,
813   A., Davies, S., Williams, A. C., Blair, A., Waterman, M. L., Tonks, A. and Darley, R. L. (2019)
814   LEF-1 drives aberrant β-catenin nuclear localization in myeloid leukemia cells.
815   Haematologica. [Online] Vol.104 (7), pp.1365–1377. Available:
816   http://www.haematologica.org/lookup/doi/10.3324/haematol.2018.202846.
817 Mosser, D. M. and Edwards, J. P. (2008) Exploring the full spectrum of macrophage activation.
818   Nature Reviews Immunology. [Online] Vol.8 (12), pp.958–969. Available:
819   http://www.nature.com/articles/nri2448.
820 Neme, A., Seuter, S. and Carlberg, C. (2016) Vitamin D-dependent chromatin association of
821   CTCF in human monocytes. Biochimica et Biophysica Acta (BBA) - Gene Regulatory
822   Mechanisms. [Online] Vol.1859 (11), pp.1380–1388. Available:
823   https://linkinghub.elsevier.com/retrieve/pii/S1874939916301833.
824 Nikukar, H., Reid, S., Tsimbouri, P. M., Riehle, M. O., Curtis, A. S. G. and Dalby, M. J. (2013)
825   Osteogenesis of Mesenchymal Stem Cells by Nanoscale Mechanotransduction. ACS Nano.
826   [Online] Vol.7 (3), pp.2758–2767. Available: https://pubs.acs.org/doi/10.1021/nn400202j.
827 Nurminen, V., Neme, A., Ryynänen, J., Heikkinen, S., Seuter, S. and Carlberg, C. (2015) The
828   transcriptional regulator BCL6 participates in the secondary gene regulatory response to
829   vitamin D. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms. [Online]
830   Vol.1849 (3), pp.300–308. Available:
831   https://linkinghub.elsevier.com/retrieve/pii/S1874939914002958.
832 Nurminen, V., Neme, A., Seuter, S. and Carlberg, C. (2019) Modulation of vitamin D signaling by
833   the pioneer factor CEBPA. Biochimica et Biophysica Acta (BBA) - Gene Regulatory
834   Mechanisms. [Online] Vol.1862 (1), pp.96–106. Available:
835   https://linkinghub.elsevier.com/retrieve/pii/S1874939918304152.
836 Nurminen, V., Seuter, S. and Carlberg, C. (2019) Primary Vitamin D Target Genes of Human
837   Monocytes. Frontiers in Physiology. [Online] Vol.10. Available:
838   https://www.frontiersin.org/article/10.3389/fphys.2019.00194/full.
839 Patel, N. R., Bole, M., Chen, C., Hardin, C. C., Kho, A. T., Mih, J., Deng, L., Butler, J.,
840   Tschumperlin, D., Fredberg, J. J., Krishnan, R. and Koziel, H. (2012) Cell Elasticity
841   Determines Macrophage Function. PLoS ONE. [Online] Vol.7 (9), p.e41024. Available:
842   http://dx.plos.org/10.1371/journal.pone.0041024.
843 Pemberton, G. D., Childs, P., Reid, S., Nikukar, H., Tsimbouri, P. M., Gadegaard, N., Curtis, A. S.
844   and Dalby, M. J. (2015) Nanoscale stimulation of osteoblastogenesis from mesenchymal
845   stem cells: nanotopography and nanokicking. Nanomedicine. [Online] Vol.10 (4), pp.547–
846   560. Available: http://www.futuremedicine.com/doi/10.2217/nnm.14.134.
847 Pennathur, S., Pasichnyk, K., Bahrami, N. M., Zeng, L., Febbraio, M., Yamaguchi, I. and Okamura,
848   D. M. (2015) The Macrophage Phagocytic Receptor CD36 Promotes Fibrogenic Pathways
849   on Removal of Apoptotic Cells during Chronic Kidney Injury. The American Journal of
850   Pathology. [Online] Vol.185 (8), pp.2232–2245. Available:

851 https://linkinghub.elsevier.com/retrieve/pii/S0002944015002758.

852 Phillip Koeffler, H., Reichel, H., Bishop, J. E. and Norman, A. W. (1985) γ-interferon stimulates
853 production of 1,25-dihydroxyvitamin D3 by normal human macrophages. Biochemical and
854 Biophysical Research Communications. [Online] Vol.127 (2), pp.596–603. Available:
855 https://linkinghub.elsevier.com/retrieve/pii/S0006291X85802023.

856 Riberdy, J. M., Newcomb, J. R., Surman, M. J., Barbosat, J. A. and Cresswell, P. (1992) HLA-DR
857 molecules from an antigen-processing mutant cell line are associated with invariant chain
858 peptides. Nature. [Online] Vol.360 (6403), pp.474–477. Available:
859 http://www.nature.com/articles/360474a0.

860 Robertson, S. N., Campsie, P., Childs, P. G., Madsen, F., Donnelly, H., Henriquez, F. L., Mackay,
861 W. G., Salmerón-Sánchez, M., Tsimbouri, M. P., Williams, C., Dalby, M. J. and Reid, S.
862 (2018) Control of cell behaviour through nanovibrational stimulation: nanokicking.
863 Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering
864 Sciences. [Online] Vol.376 (2120), p.20170290. Available:
865 https://royalsocietypublishing.org/doi/10.1098/rsta.2017.0290.

866 Schwende, H., Fitzke, E., Ambs, P. and Dieter, P. (1996) Differences in the state of
867 differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D 3.
868 Journal of Leukocyte Biology. [Online] Vol.59 (4), pp.555–561. Available:
869 http://doi.wiley.com/10.1002/jlb.59.4.555.

870 Seuter, S., Neme, A. and Carlberg, C. (2016) Epigenome-wide effects of vitamin D and their
871 impact on the transcriptome of human monocytes involve CTCF. Nucleic Acids Research.
872 [Online] Vol.44 (9), pp.4090–4104. Available: https://academic.oup.com/nar/article-
873 lookup/doi/10.1093/nar/gkv1519.

874 Seuter, S., Neme, A. and Carlberg, C. (2017) Epigenomic PU.1-VDR crosstalk modulates vitamin
875 D signaling. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms. [Online]
876 Vol.1860 (4), pp.405–415. Available:
877 https://linkinghub.elsevier.com/retrieve/pii/S1874939916302929.

878 Seuter, S., Neme, A. and Carlberg, C. (2018) ETS transcription factor family member GABPA
879 contributes to vitamin D receptor target gene regulation. The Journal of Steroid
880 Biochemistry and Molecular Biology. [Online] Vol.177, pp.46–52. Available:
881 https://linkinghub.elsevier.com/retrieve/pii/S0960076017302200.

882 Shao, L. and Sperber, K. (2002) Impaired Regulation of HLA-DR Expression in Human
883 Immunodeficiency Virus-Infected Monocytes. Clinical and Vaccine Immunology. [Online]
884 Vol.9 (4), pp.739–746. Available: https://cvi.asm.org/content/9/4/739.

885 Shi, C. and Pamer, E. G. (2011) Monocyte recruitment during infection and inflammation.
886 Nature Reviews Immunology. [Online] Vol.11 (11), pp.762–774. Available:
887 http://www.nature.com/articles/nri3070.

888 Sloan, V. S., Cameron, P., Porter, G., Gammon, M., Amaya, M., Mellins, E. and Zaller, D. M.
889 (1995) Mediation by HLA-DM of dissociation of peptides from HLA-DR. Nature. [Online]
890 Vol.375 (6534), pp.802–806. Available: http://www.nature.com/articles/375802a0.

891 Solis, A. G., Bielecki, P., Steach, H. R., Sharma, L., Harman, C. C. D., Yun, S., de Zoete, M. R.,
892 Warnock, J. N., To, S. D. F., York, A. G., Mack, M., Schwartz, M. A., Dela Cruz, C. S., Palm, N.
893 W., Jackson, R. and Flavell, R. A. (2019) Mechanosensation of cyclical force by PIEZO1 is
894 essential for innate immunity. Nature. [Online] Vol.573 (7772), pp.69–74. Available:

895   http://www.nature.com/articles/s41586-019-1485-8.

896 Thiele, A., Wasner, M., Müller, C., Engeland, K. and Hauschildt, S. (2001) Regulation and

897   Possible Function of β-Catenin in Human Monocytes. The Journal of Immunology. [Online]

898   Vol.167 (12), pp.6786–6793. Available:

899   http://www.jimmunol.org/lookup/doi/10.4049/jimmunol.167.12.6786.

900 Tickenbrock, L. (2006) Wnt signaling regulates transendothelial migration of monocytes. Journal

901   of Leukocyte Biology. [Online] Vol.79 (6), pp.1306–1313. Available:

902   http://doi.wiley.com/10.1189/jlb.0905539.

903 Tokuda, N. and Levy, R. B. (1996) 1,25-Dihydroxyvitamin D3 Stimulates Phagocytosis but

904   Suppresses HLA-DR and CD13 Antigen Expression in Human Mononuclear Phagocytes.

905   Experimental Biology and Medicine. [Online] Vol.211 (3), pp.244–250. Available:

906   http://ebm.sagepub.com/lookup/doi/10.3181/00379727-211-43967.

907 Tsimbouri, P. (2015) Adult Stem Cell Responses to Nanostimuli. Journal of Functional

908   Biomaterials. [Online] Vol.6 (3), pp.598–622. Available: http://www.mdpi.com/2079-

909   4983/6/3/598.

910 Tsuchiya, S., Gota, Y., Okumura, H., Nakae, S., Konno, T., Tada, K. and Kobayashi, Y. (1982)

911   Induction of Maturation in Cultured Human Monocytic Leukemia Cells by a Phorbol

912   Diester. Cancer Research. Vol.42 (4), pp.1530–6.

913 Ulbricht, T., Alzrigat, M., Horch, A., Reuter, N., von Mikecz, A., Steimle, V., Schmitt, E., Krämer,

914   O. H., Stamminger, T. and Hemmerich, P. (2012) PML promotes MHC class II gene

915   expression by stabilizing the class II transactivator. The Journal of Cell Biology. [Online]

916   Vol.199 (1), pp.49–63. Available: https://rupress.org/jcb/article/199/1/49/37158/PML-

917   promotes-MHC-class-II-gene-expression-by.

918 Wöbke, T. K., Sorg, B. L. and Steinhilber, D. (2014) Vitamin D in inflammatory diseases. Frontiers

919   in Physiology. [Online] Vol.5. Available:

920   http://journal.frontiersin.org/article/10.3389/fphys.2014.00244/abstract.

921 Woo, M.-S., Yang, J., Beltran, C. and Cho, S. (2016) Cell Surface CD36 Protein in

922   Monocyte/Macrophage Contributes to Phagocytosis during the Resolution Phase of

923   Ischemic Stroke in Mice. Journal of Biological Chemistry. [Online] Vol.291 (45), pp.23654–

924   23661. Available: http://www.jbc.org/lookup/doi/10.1074/jbc.M116.750018.

925 Yamamoto, K., Ikeda, U. and Shimada, K. (2003) Role of Mechanical Stress in Monocytes /

926   Macrophages: Implications for Atherosclerosis. Current Vascular Pharmacology. [Online]

927   Vol.1 (3), pp.315–319. Available:

928   http://www.eurekaselect.com/openurl/content.php?genre=article&issn=1570-

929   1611&volume=1&issue=3&spage=315.

930 Yang, C.-Y., Leung, P. S. C., Adamopoulos, I. E. and Gershwin, M. E. (2013) The Implication of

931   Vitamin D and Autoimmunity: a Comprehensive Review. Clinical Reviews in Allergy &

932   Immunology. [Online] Vol.45 (2), pp.217–226. Available:

933   http://link.springer.com/10.1007/s12016-013-8361-3.

934 Yang, M., Ma, B., Shao, H., Clark, A. M. and Wells, A. (2016) Macrophage phenotypic subtypes

935   diametrically regulate epithelial-mesenchymal plasticity in breast cancer cells. BMC

936   Cancer. [Online] Vol.16 (1), p.419. Available:

937   https://bmccancer.biomedcentral.com/articles/10.1186/s12885-016-2411-1.

938 Zanoni, I., Ostuni, R., Marek, L. R., Barresi, S., Barbalat, R., Barton, G. M., Granucci, F. and Kagan,

939   J. C. (2011) CD14 Controls the LPS-Induced Endocytosis of Toll-like Receptor 4. Cell.

940   [Online] Vol.147 (4), pp.868–880. Available:

941   https://linkinghub.elsevier.com/retrieve/pii/S0092867411012219.

942   Zhang, D. E., Hetherington, C. J., Gonzalez, D. A., Chen, H. M. and Tenen, D. G. (1994) Regulation

943   of CD14 expression during monocytic differentiation induced with 1 alpha,25-

944   dihydroxyvitamin D3. Journal of immunology (Baltimore, Md. : 1950). Vol.153 (7),

945   pp.3276–84.

946   Zhao Q, L, W., J, H. and H., L. (2016) [Role of nuclear factor of activated T cells-2 in high mobility

947   protein box-1 release in human monocytic THP-1 cells in vitro]. Journal of Southern

948   Medical University (Chinese). [Online] Vol.36 (1), pp.8–12. Available:

949   https://www.ncbi.nlm.nih.gov/pubmed/26806731.

950   Zhong, M., Komarova, Y., Rehman, J. and Malik, A. B. (2018) Mechanosensing Piezo channels in

951   tissue homeostasis including their role in lungs. Pulmonary Circulation. [Online] Vol.8 (2),

952   p.204589401876739. Available:

953   http://journals.sagepub.com/doi/10.1177/2045894018767393.

954

Figure 1

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

A) mRNA regulation in response to stimulation with 50nM of 1,25-dihydroxyvitamin D3. B) mRNA regulation in response to 1000Hz vibrations (amplitude 30 - 60 nm). C) mRNA regulation in response to the combined vitamin D (50nM) and 1000Hz (30 - 60 nm amplitude) vibrations. Data presented as mean of 4 replicates ± SEM. Statistical analysis between stimulated and control values was assessed by unpaired T test with Welch's correction. P values lower than 0.05 were considered statistically significant.

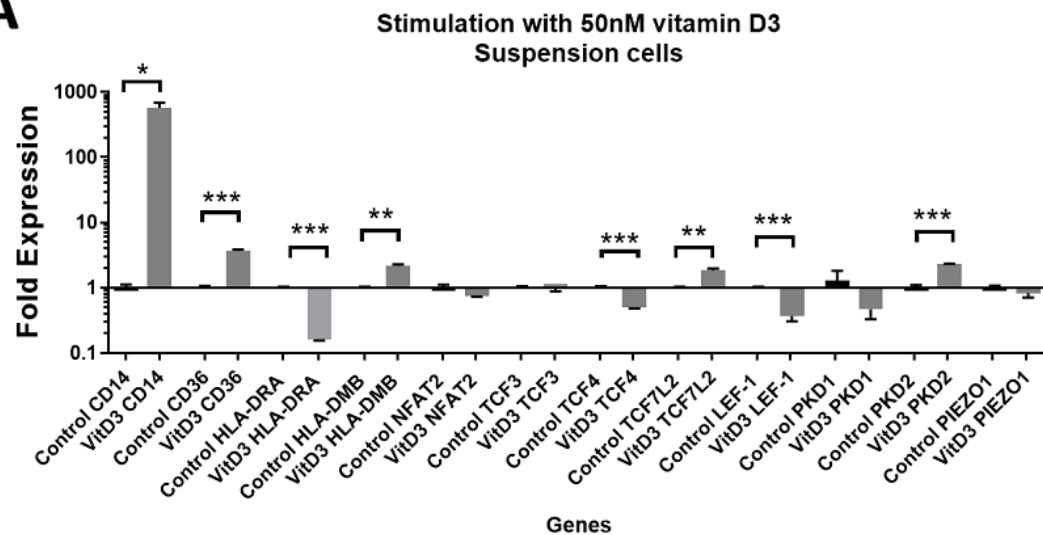
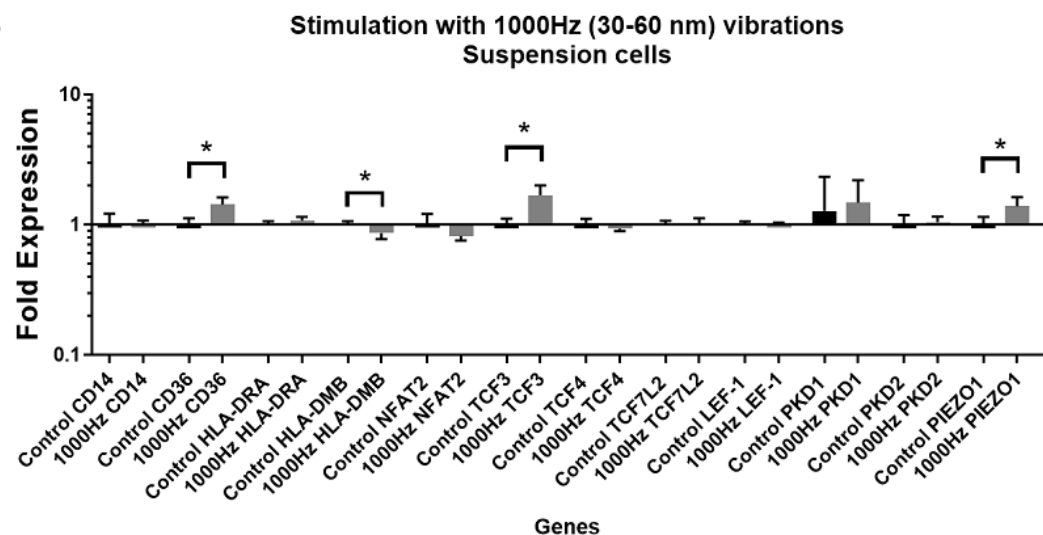
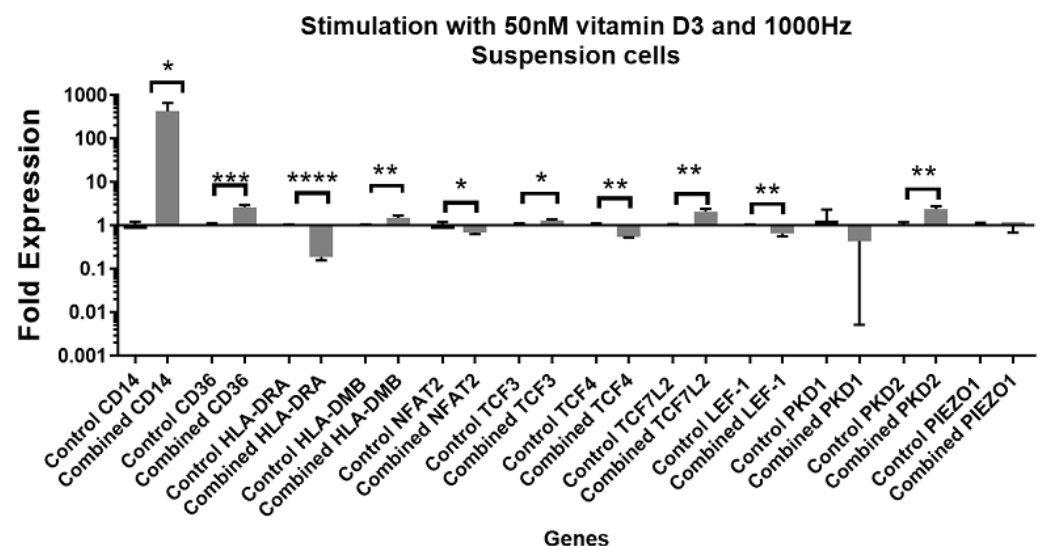



Figure 2

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

A) mRNA regulation in response to stimulation with 50nM of 1,25-dihydroxyvitamin D3. B) mRNA regulation in response to 1000Hz vibrations (amplitude 30 - 60 nm). C) mRNA regulation in response to the combined vitamin D (50nM) and 1000Hz (30 - 60 nm amplitude) vibrations. Data presented as mean of 4 replicates ± SEM. Statistical analysis between stimulated and control values was assessed by unpaired T test with Welch's correction. P values lower than 0.05 were considered statistically significant.

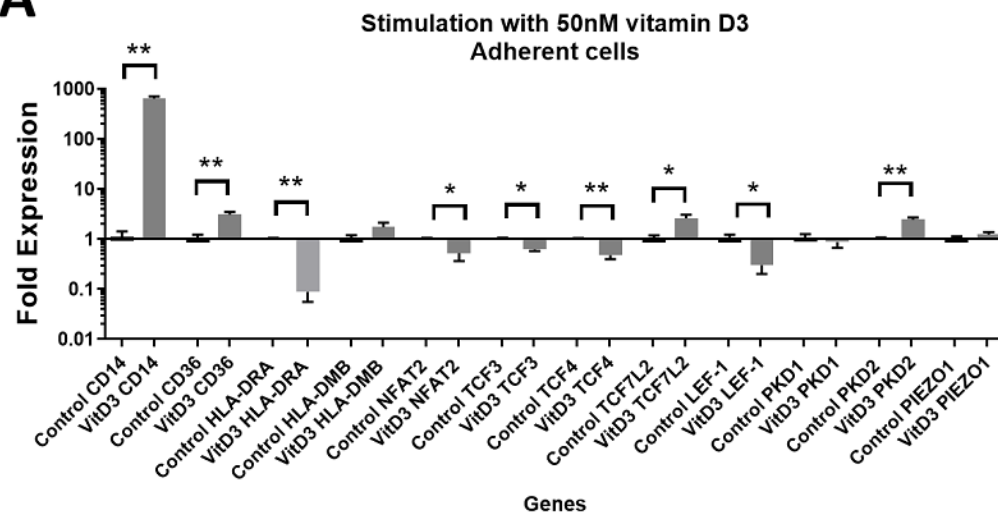
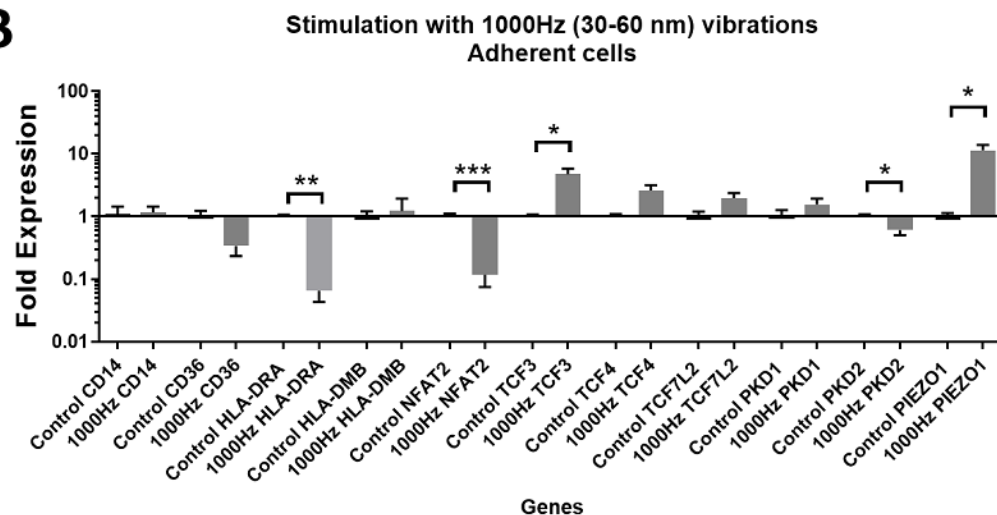
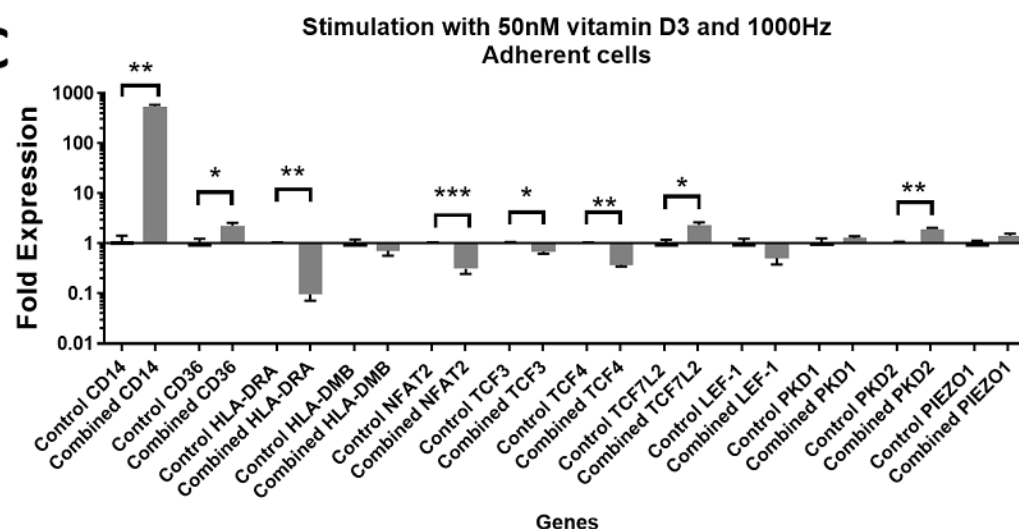
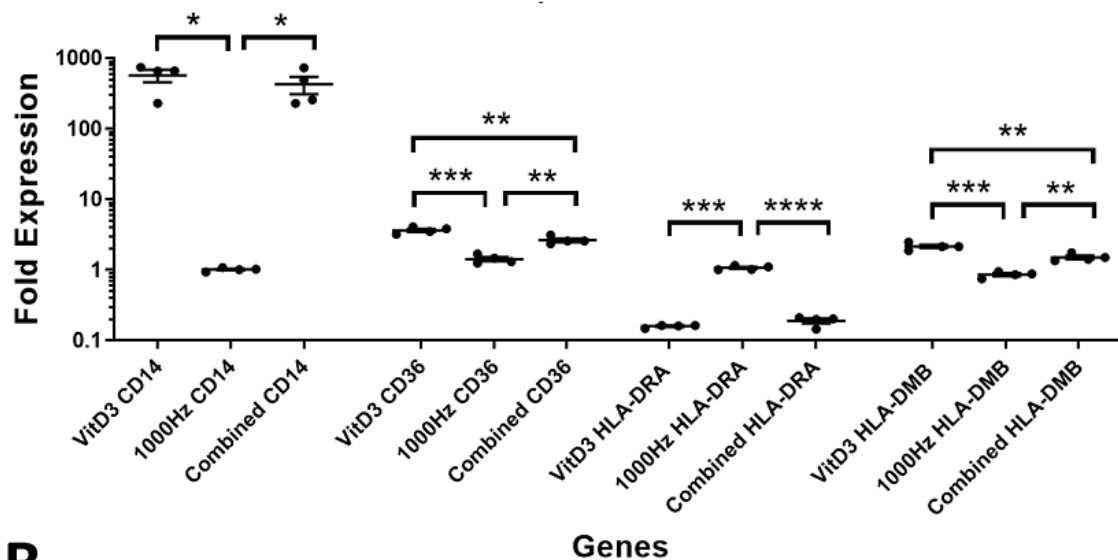
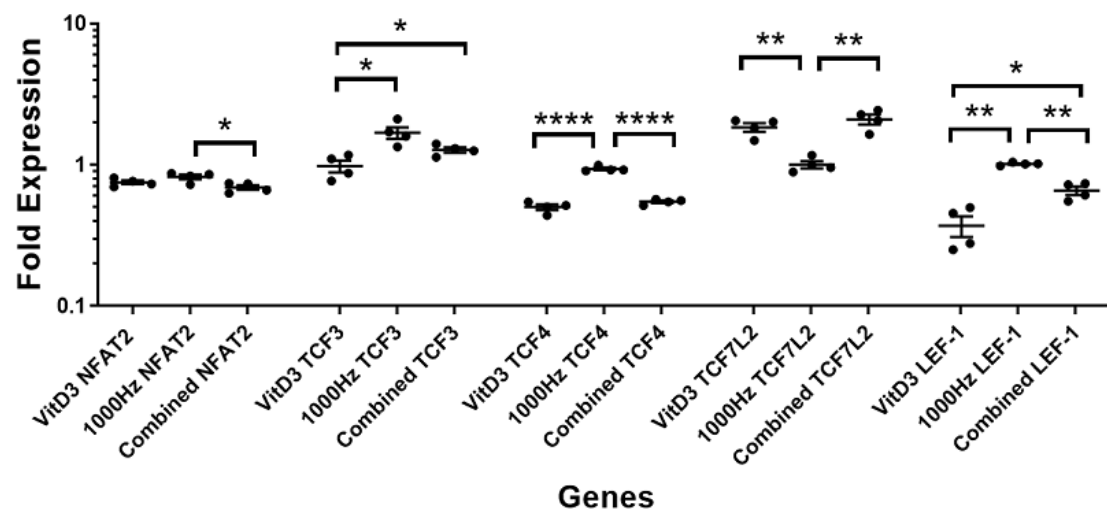



Figure 3

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 unpaired T test with Welch's correction. P values lower than 0.05 were considered statistically significant. Genes investigated encode for markers of macrophage differentiation (A), transcription factors (B), and mechanosensors (C).

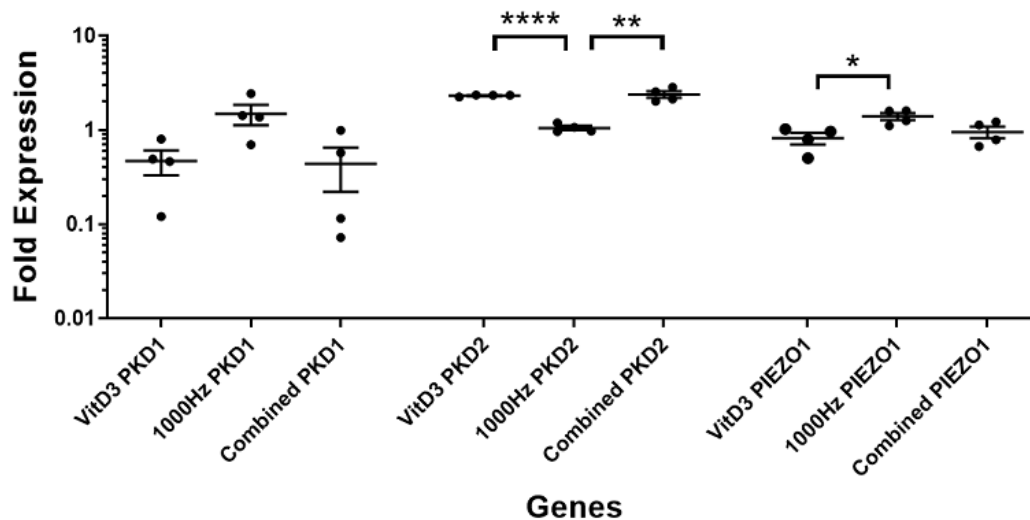
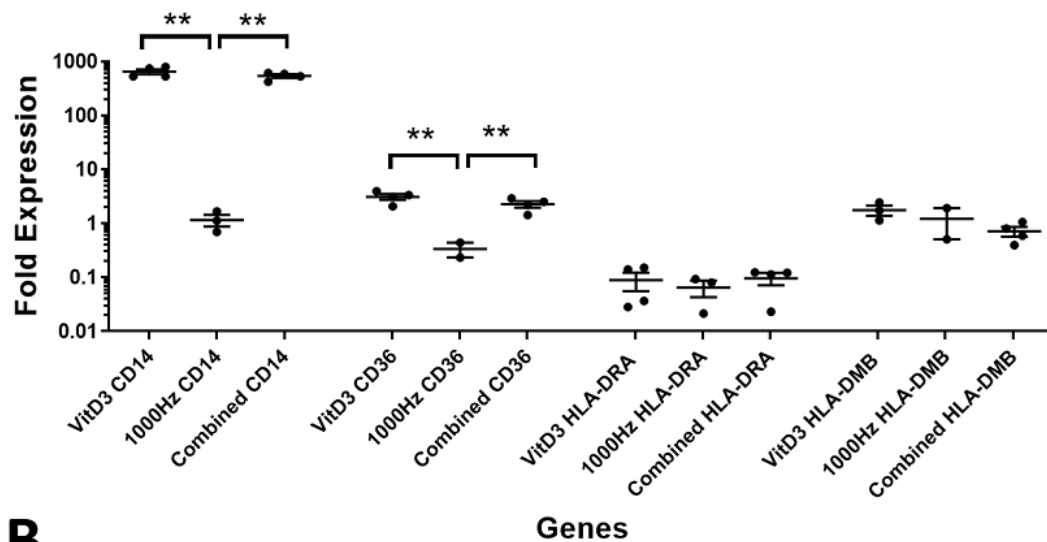
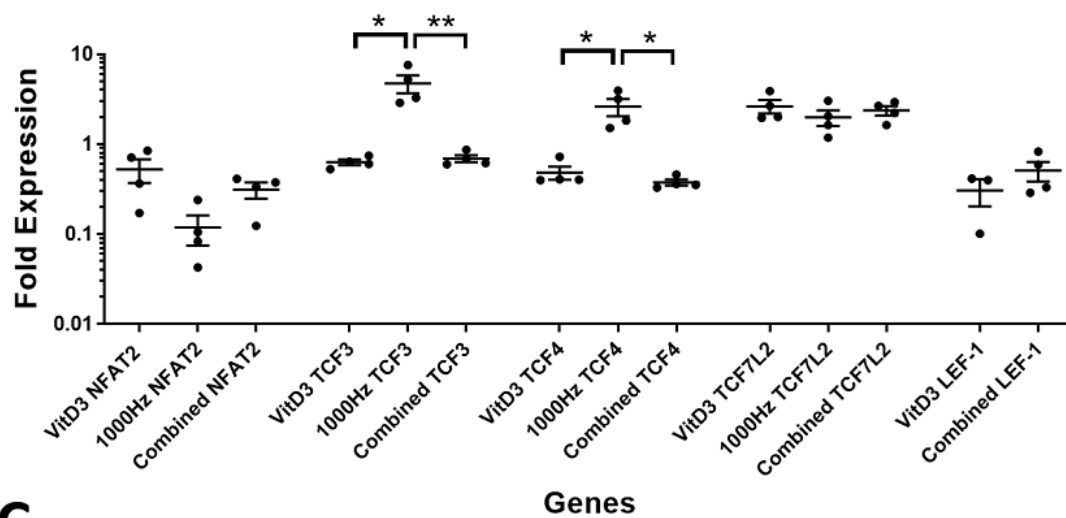
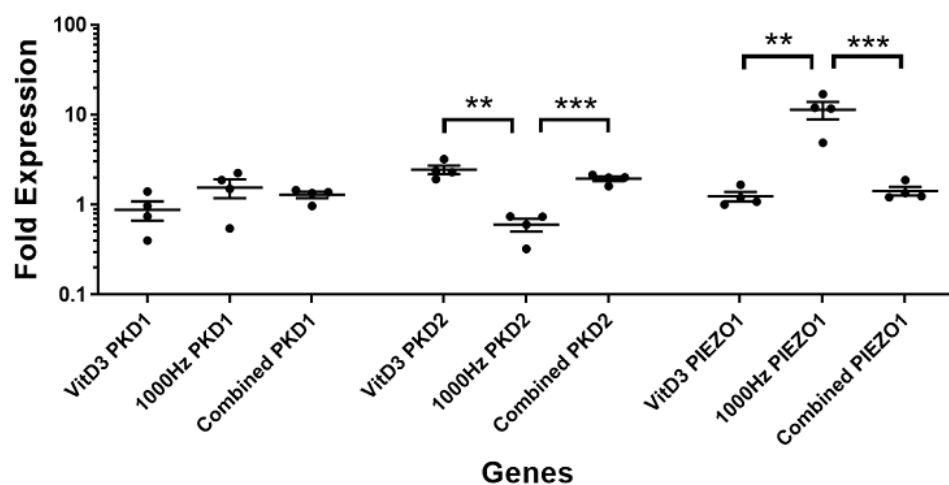



Figure 4

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

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

Expression of genes in stimulated suspension THP-1 cells compared to the unstimulated suspension cells at 72 hours.

Statistical analysis was performed using unpaired T test with Welch's correction. Fold change

($2^{-\Delta\Delta Ct}$) 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**	1.69	0.0182

		macrophages)		
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**

Expression of genes in stimulated adherent THP-1 cells compared to the unstimulated adherent cells at 72 hours.

Statistical analysis was performed using unpaired T test with Welch's correction. Fold change

$(2^{-\Delta\Delta Ct})$ 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

50 nM 1,25(OH)2 D3 + Vibrations 1000Hz (30 - 60 nm) (72 hours)	*NFAT2*	**Transcription factor (undefined roles in macrophages)**	**0.12**	**0.0004**
	TCF3	**Transcription factor (unknown roles in macrophages)**	**4.73**	**0.04**
	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**

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 unpaired 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)