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Impact of salt and the osmoprotective transcription factor NFAT-5 on macrophages during mechanical strain

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Abstract

Myeloid cells regulate bone density in response to increased salt (NaCl) intake via the osmoprotective transcription factor, nuclear factor of activated T cells-5 (NFAT-5). Because orthodontic tooth movement (OTM) is a pseudoinflammatory immunological process, we investigated the influence of NaCl and NFAT-5 on the expression pattern of macrophages in a model of simulated OTM. RAW264.7 macrophages were exposed for 4 h to 2 g cm^{-2} compressive or 16% tensile or no mechanical strain (control), with or without the addition of 40 mM NaCl. We analyzed the expression of inflammatory genes and proteins [tumor necrosis factor (TNF), interleukin (IL)-6 and prostaglandin endoperoxide synthase-2 (*Ptgs-2*)/prostaglandin E2 (PG-E2)] by real-time-quantitative PCR and ELISA. To investigate the role of NFAT-5 in these responses, NFAT-5 was both constitutively expressed and silenced. Salt and compressive strain, but not tensile strain increased the expression of NFAT-5 and most tested inflammatory factors in macrophages. NaCl induced the expression of *Ptgs-2*/PG-E2 and TNF, whereas secretion of IL-6 was inhibited. Similarly, a constitutive expression of NFAT-5 reduced IL-6 expression, while increasing *Ptgs-2*/PG-E2 and TNF expression. Silencing of NFAT-5 upregulated IL-6 and reduced *Ptgs-2*/PG-E2 and TNF expression. Salt had an impact on the expression profile of macrophages as a reaction to compressive and tensile strain that occur during OTM. This was mediated via NFAT-5, which surprisingly also seems to play a regulatory role in mechanotransduction of compressive strain. Sodium accumulation in the periodontal ligament caused by dietary salt consumption might propagate local osteoclastogenesis via increased local inflammation and thus OTM velocity, but possibly also entail side effects such as dental root resorptions or periodontal bone loss.

INTRODUCTION

A key aspect of orthodontic treatment is the correction of misplaced teeth by orthodontic appliances. This correction has great medical importance, as malocclusions of teeth are associated with increased caries[1] and gingivitis prevalence.[2] Therefore, orthodontic therapy has a prophylactic function for the development and progression of these diseases.

To achieve orthodontic tooth movement (OTM), a mechanical force is transferred to the tooth in direction of movement by means of removable or fixed orthodontic appliances.[3] This applied force provokes formation of tensile and pressure zones in the

periodontal ligament, a fibrous and cell-rich connective tissue that anchors the tooth in the alveolar socket of the jawbone. Within the first hours after onset of orthodontic treatment, a broad spectrum of proinflammatory mediators is released by periodontal ligament fibroblasts and lymphocytes.[4–8] In tension zones deposition of new bone on the alveolar surface occurs, whereas in pressure zones bone resorption is observed.[45] These effects are not only mediated by periodontal ligament fibroblasts, but also modulated by cells of the innate immune system.[9] Macrophages are an integral part of innate immunity and play a central role in inflammatory responses and host defense. In addition, these cells regulate tissue homeostasis in various pathophysiological processes, including innate and adaptive immune responses, wound healing, hematopoiesis and malignancy.[10] Despite the importance of orthodontic treatment for oral health, many aspects of orthodontic therapy have so far been not investigated. Because OTM represents a local sterile inflammatory, immunological process,[5] there are numerous possibilities of modulation by the metabolism and the immune system, with tooth movement being affected by systemic and exogenous influences.[11,12] Apart from pharmaceuticals, a modulation of OTM is also expected from certain lifestyle habits such as diet.

Crucially, Western nutritional deficiencies are considered to cause inflammation and to reinforce chronic diseases such as high blood pressure.[13–16] Nutrition also has an impact on the oral microflora and it has been shown that an oral health-optimized diet results in a reduction of clinical inflammatory parameters such as gingival index and periodontal status.[17] Even electrolytes such as sodium and potassium are consumed in modern society to a high degree as a dietary supplement and can therefore be pharmacologically and immunologically effective in appropriate doses via the electrolyte balance and their local tissue concentration.[14,18] These phenomena also occur in tissues of the oral cavity, in particular the oral mucosa, the gingiva and the periodontal ligament as components of the periodontal ligament.[19] Salt (NaCl) exposure is known to promote osteoclast formation during pressure application in periodontal ligament fibroblasts, indicating a direct effect on tooth movement velocity.[19] Observed Na^+ accumulation, however, can also impact on the activity of immune cells such as macrophages.[18] It is thus to be assumed that the local sodium content in the periodontal ligament could also have an influence on the OTM-induced sterile inflammatory reaction. The osmoprotective transcription factor, nuclear factor of activated T cells-5 (NFAT-5), is known to be involved in many regulatory pathways during salt exposure.[13,14,18,20]

The impact of immunomodulatory properties of electrolytes on macrophages during OTM has not been studied. In addition to the formulation of first preventative recommendations for diet during orthodontic therapy, the exploration of these relationships may allow the targeted use of electrolyte solutions as adjuvant orthodontic therapeutics, especially in the case of a conceivable local topical application in the periodontal ligament. During OTM, macrophages located in the periodontal ligament are also subjected to mechanical tensile and compressive stress. The response of macrophages to these stimuli will be investigated in this study under normal salt and high salt conditions.

RESULTS

Effects of salt on inflammatory cytokine expression during tensile strain

First, we investigated the impact of salt (NaCl) on inflammatory cytokine expression in RAW264.7 macrophages and bone-marrow-derived macrophages (BMMs; Supplementary figure 1) during tensile strain. We focused on the expression of tumor necrosis factor (*Tnf*), interleukin-6 (*Il-6*), prostaglandin endoperoxide synthase-2 (*Ptgs-2*) and prostaglandin E2 (PG-E2), which are critically involved in the regulation of OTM by controlling and promoting among other things RANKL and OPG expression, leading to increased osteoclastogenesis required for OTM.[5,9,21,22] In RAW264.7 macrophages tensile strain increased *Tnf* messenger RNA (mRNA; $P = 0.0103$) and protein expression significantly ($P = 0.0027$; Figure 1a). NaCl treatment elevated expression of TNF at the mRNA ($P = 0.0002$) and protein level ($P = 0.0040$; Figure 1a), whereas a combination of mechanical tensile strain and salt did not further enhance *Tnf* expression (Figure 1a). These effects of tensile loading on *Tnf* gene expression were confirmed in BMMs, which show similar results (Supplementary figure 1a).

IL-6 expression was increased at the mRNA ($P = 0.0004$) and protein ($P < 0.0001$) level after applying tensile strain onto RAW264.7 macrophages (Figure 1b) and BMMs (Supplementary figure 1b). This effect of isotropic stretching was inhibited by NaCl treatment at the gene ($P = 0.0353$) and protein expression level ($P = 0.0004$; Figure 1b). By contrast, gene expression of *Ptgs-2* was increased only after NaCl addition ($P = 0.0008$), without any detectable effect of tensile strain ($P = 0.8073$; Figure 1c). In line with that, we detected a salt-dependent increase in PG-E2 secretion after NaCl treatment ($P = 0.0005$; Figure 1c). Using

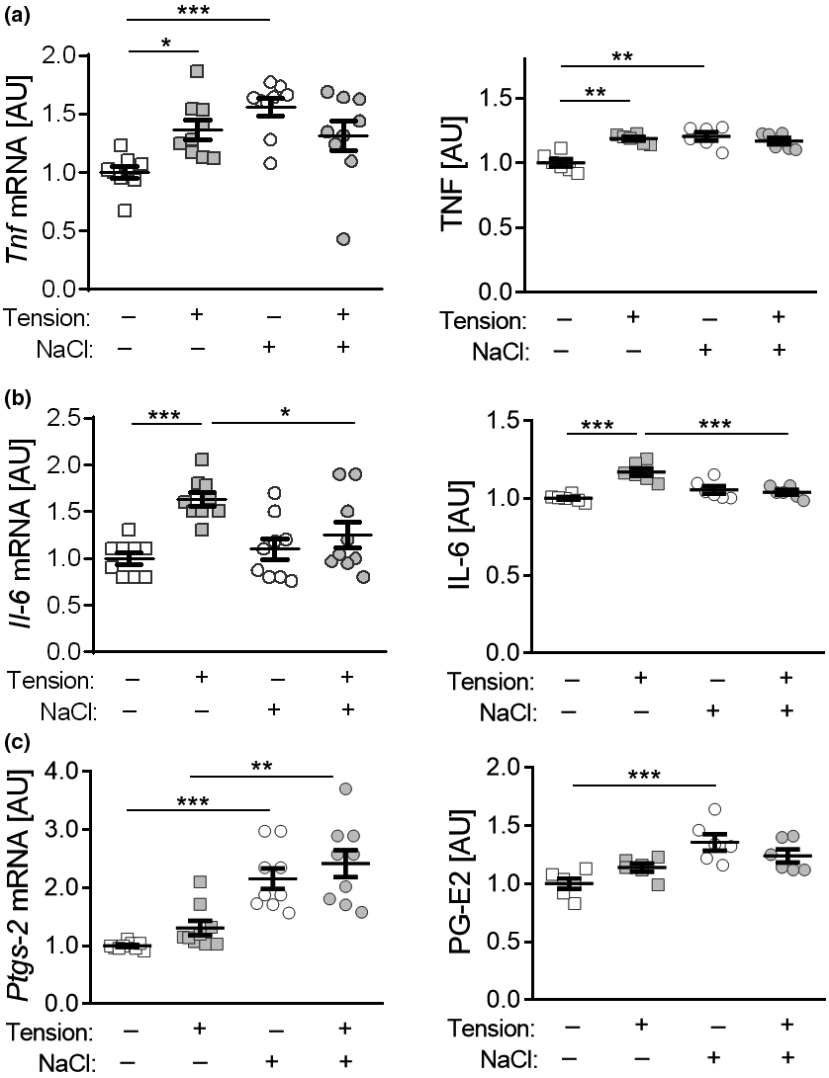

Figure 1. Gene and protein expression of **(a)** TNF, **(b)** IL-6 and **(c)** *Ptgs-2*/PG-E2 after tensile strain during normal or high salt (NaCl) conditions for 4 h. Data were analyzed by ANOVA followed by Holm–Šídák or Games–Howell multiple comparison tests. AU, arbitrary units; IL, interleukin; mRNA, messenger RNA; PG-E2, prostaglandin E2; Ptgs-2, prostaglandin endoperoxide synthase-2; TNF, tumor necrosis factor. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

BMMs we observed similar effects on *Ptgs-2* gene expression (Supplementary figure 1c).

Effects of NaCl on inflammatory cytokine expression during compressive strain

Next, we performed experiments with pressure application in RAW264.7 macrophages and BMMs (Supplementary figure 1) and investigated inflammatory cytokine expression. In RAW264.7 macrophages, pressure application significantly increased TNF expression on the mRNA ($P < 0.0001$) and protein level ($P = 0.0027$), whereas the addition of NaCl did not impact on *Tnf* gene expression ($P = 0.9421$), but on TNF secretion ($P = 0.0012$; Figure 2a). Like in RAW264.7 macrophages, we observed increased *Tnf* gene expression in BMMs (Supplementary figure 1). *Il-6* gene expression was enhanced by compressive strain in RAW264.7 macrophages ($P = 0.0182$; Figure 2b) and BMMs (Supplementary figure 1). However, addition of NaCl attenuated this pressure-induced effect ($P = 0.0358$; Figure 2b). According to gene expression data, IL-6 secretion was elevated after pressure application ($P < 0.0001$) and this effect was inhibited by addition of NaCl ($P < 0.0001$; Figure 2b). Without additional pressure application, NaCl did not impact on IL-6 gene ($P = 0.3092$) or protein expression ($P = 0.4816$).

Pressure treatment increased *Ptgs-2* gene expression ($P < 0.0001$) and PG-E2 protein secretion in RAW264.7 macrophages ($P < 0.0001$; Figure 2c) and BMMs (Supplementary figure 1c). Treatment with NaCl further significantly enhanced *Ptgs-2* gene expression without ($P = 0.0397$) and with simultaneous pressure application ($P < 0.0001$). However, PG-E2 protein secretion was only further elevated after addition of NaCl without pressure application ($P = 0.0269$; Figure 2c).

Impact of mechanical strain and NaCl on NFAT-5 expression

Next we focused on NFAT-5 expression during mechanical strain and salt exposure. As expected, NaCl exposure (high salt conditions) increased NFAT-5 expression at the mRNA ($P < 0.0001$) and protein level ($P < 0.0001$; Figure 3a, b). Under control conditions, tensile strain did not impact on NFAT-5 expression in RAW264.7 macrophages ($P = 0.9773$; Figure 3a) and BMMs (Supplementary figure 1d). Additional NaCl exposure during tensile strain reduced *Nfat-5* mRNA expression ($P = 0.004$), whereas NFAT-5 protein expression remained unaffected ($P = 0.0635$; Figure 3a).

By contrast, compressive strain enhanced NFAT-5 expression in RAW264.7 macrophages ($P = 0.0136$, Figure 3b) and BMMs (Supplementary figure 1d). Without pressure application salt exposure increased *Nfat-5* gene ($P = 0.0035$) and protein expression ($P = 0.0015$) on conventional polystyrene plates.

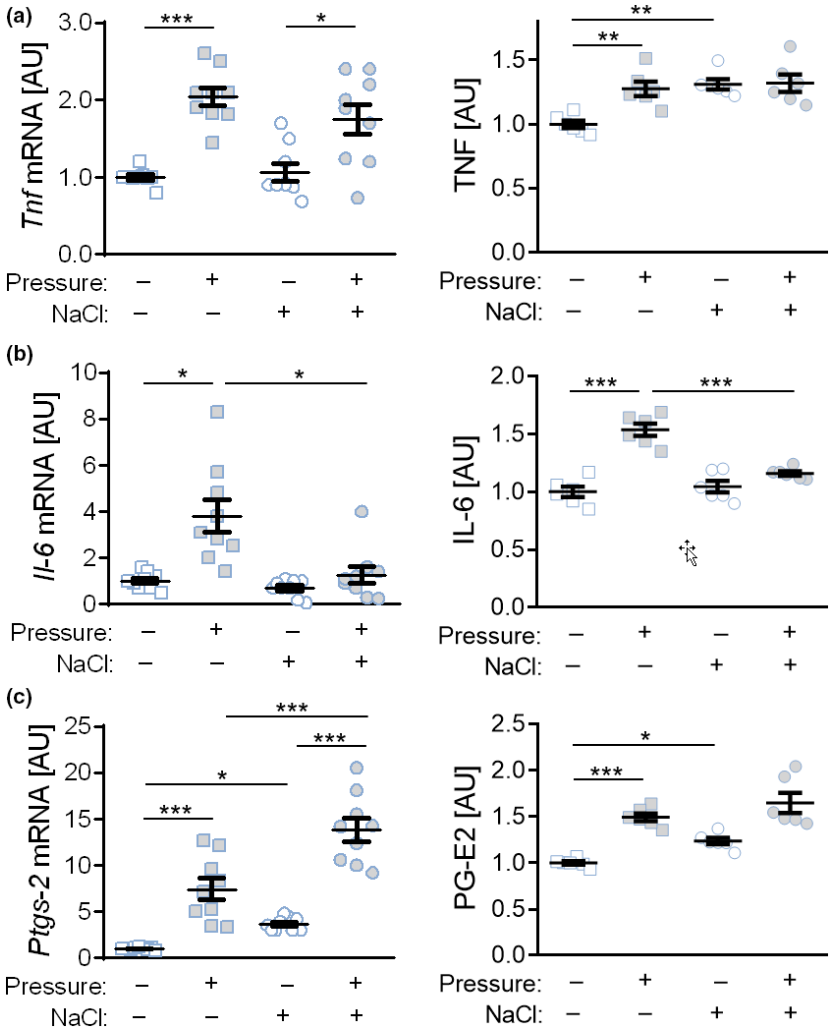

Figure 2. Gene and protein expression of **(a)** TNF, **(b)** IL-6 and **(c)** *Ptgs-2*/PG-E2 after compressive strain during normal or high salt (NaCl) conditions for 4 h. Data were analyzed by ANOVA followed by Holm–Šídák or Games–Howell multiple comparison tests. AU, arbitrary units; IL, interleukin; mRNA, messenger RNA; PG-E2, prostaglandin E2; Ptgs-2, prostaglandin endoperoxide synthase-2; TNF, tumor necrosis factor. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

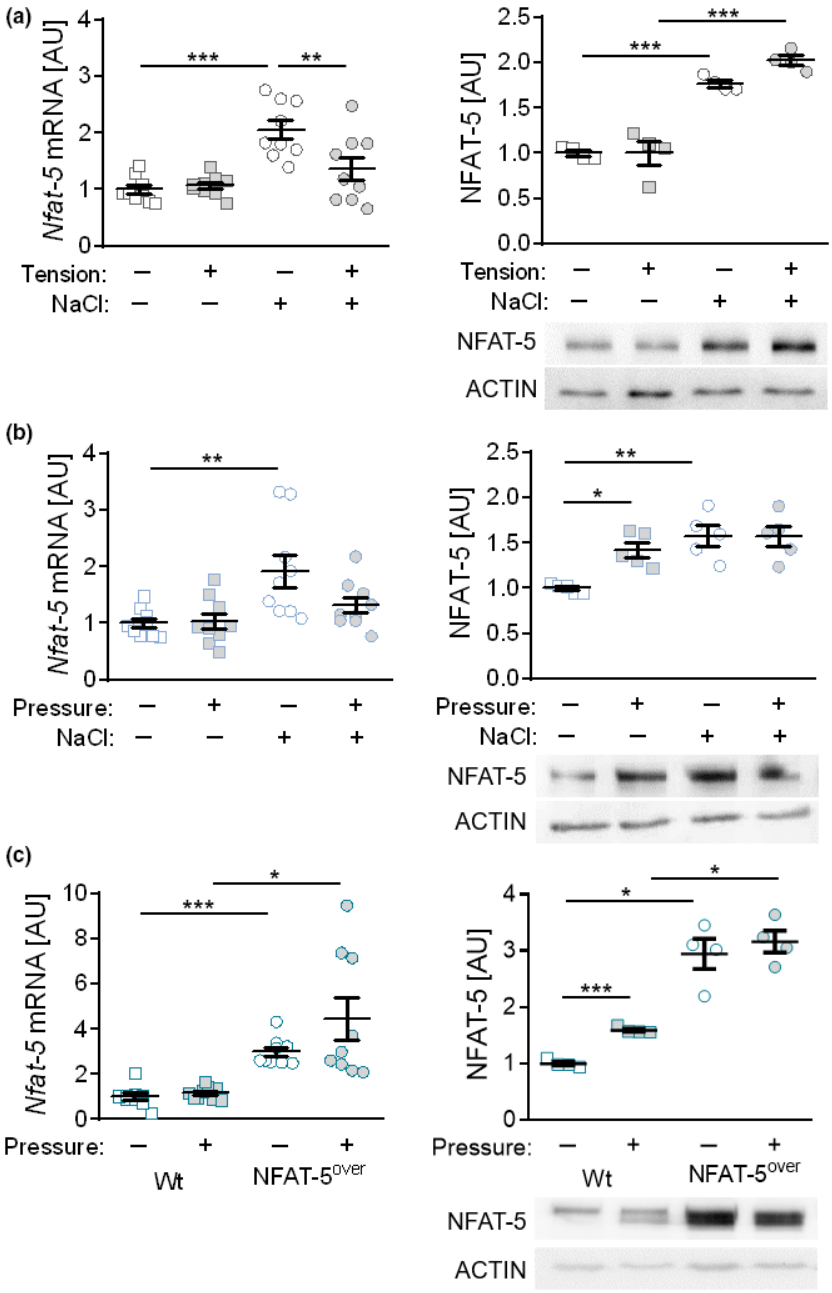

Figure 3. NFAT-5 gene and protein expression after **(a)** tensile strain or **(b)** compressive strain of RAW264.7 macrophages during normal or high salt (NaCl) conditions for 4 h. **(c)** NFAT-5 gene and protein expression in RAW264.7 macrophages [wild type (Wt)] or NFAT-5-overexpressing macrophages (NFAT-5^{over}). One representative immunoblot each. Data were analyzed by ANOVA followed by Holm–Šídák or Games–Howell multiple comparison tests. AU, arbitrary units; mRNA, messenger RNA; NFAT-5, nuclear factor of activated T cells-5; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Compressive strain reduced this NaCl-induced effect (Figure 3b).

To further investigate the role of NFAT-5 during compressive strain, we performed experiments with NFAT-5-overexpressing (NFAT-5^{over}) macrophages and compared them with conventional RAW264.7 macrophages (wild type). As expected, we detected enhanced *Nfat-5* mRNA expression without ($P < 0.0001$)

and with additional compressive strain ($P = 0.0225$; Figure 3c). Accordingly, NFAT-5 protein expression was elevated in NFAT-5^{over} macrophages without ($P = 0.0150$) and with mechanical compressive strain ($P = 0.0107$). Of note, we again detected enhanced NFAT-5 protein levels after compressive strain on wild-type macrophages ($P < 0.0001$; Figure 3c).

Effects of NFAT-5 overexpression on inflammatory cytokine expression during compressive strain

As tensile strain did not impact on NFAT-5 expression, we focused on pressure application with NFAT-5-overexpressing macrophages (NFAT-5^{over}). We detected increased *Tnf* gene expression ($P = 0.0014$) and protein secretion ($P < 0.0001$) in NFAT-5^{over} cells not subjected to pressure application (Figure 4a), indicating a promotional role of NFAT-5 in *Tnf* expression and secretion in macrophages. Compressive strain reduced *Tnf* gene expression ($P = 0.0004$), whereas TNF protein secretion was further elevated ($P = 0.0093$; Figure 4a). NFAT-5 overexpression had no effect on *Il-6* gene expression without compressive strain ($P = 0.5213$). On the protein level, however, we detected reduced IL-6 protein secretion in NFAT-5^{over} macrophages ($P = 0.0002$; Figure 4b). Combined with mechanical compressive strain NFAT-5 overexpression inhibited *Il-6* gene expression ($P < 0.0001$) and protein secretion ($P = 0.0002$), indicating a regulatory role of NFAT-5 in IL-6 expression. Gene expression of *Ptgs-2* was increased with compressive strain ($P = 0.0003$) and NFAT-5 overexpression under control conditions ($P < 0.0001$; Figure 4c). Accordingly, we detected increased PG-E2 secretion with pressure ($P = 0.0378$) or NFAT-5 overexpression ($P = 0.0491$, Figure 4c), which was expected, as *Ptgs-2* is a common NFAT-5 target gene.

Effects of Nfat-5 silencing on inflammatory cytokine expression during compressive strain

To further determine the role of NFAT-5, we silenced NFAT-5 expression in RAW264.7 macrophages using small interfering RNA (siRNA) electroporation. We detected reduced *Nfat-5* gene expression after compressive strain ($P < 0.0001$), NaCl addition ($P = 0.0003$) and salt combined with pressure ($P = 0.0058$; Figure 5a). Accordingly, NFAT-5 protein expression was significantly reduced with compressive strain ($P = 0.0031$) after transfection with *Nfat-5*-specific siRNA, proving that the silencing worked.

Tnf gene expression was not affected by *Nfat-5* silencing (Figure 5b). However, *Nfat-5* silencing reduced TNF secretion in all investigated conditions (Figure 5b). Of note, we still detected an increase of TNF protein expression during compressive strain, indicating that NFAT-5 is not involved in mechanotransductive regulation of *Tnf* expression. Surprisingly, TNF protein secretion was no longer elevated after salt exposure without ($P > 0.9999$) and with compressive strain ($P = 0.4435$; Figure 5b).

Without pressure application *Il-6* gene expression ($P = 0.0080$) and secretion ($P = 0.0244$) were significantly enhanced by *Nfat-5* silencing (Figure 5c). Again, salt exposure reduced the effect of compressive strain on *Il-6* at the gene ($P = 0.019$) and protein level ($P = 0.0163$). This effect was abolished by *Nfat-5* silencing (Figure 5c). After *Nfat-5* siRNA treatment macrophages failed to increase IL-6 secretion upon compressive strain ($P = 0.2857$), indicating a role of NFAT-5 in mechanotransductive regulation of IL-6 expression.

Ptgs-2 gene expression was elevated upon mechanical compressive strain under all investigated conditions (Figure 5d). Only combined salt and pressure exposure showed reduced *Ptgs-2* gene expression after *Nfat-5* siRNA silencing ($P = 0.004$). Surprisingly, PG-E2 secretion was reduced under all investigated conditions with Nfat-5 silencing, indicating a regulatory role of NFAT-5 in PG-E2 expression during salt exposure and mechanical compressive strain (Figure 5d).

DISCUSSION

Mechanical strain occurring in the periodontal ligament of teeth during OTM, which impacts on local cells such as periodontal ligament fibroblasts, macrophages and other immune cells such as lymphocytes, can be distinguished in compressive and tensile strain. While tensile strain only increased TNF and IL-6 secretion in macrophages, compressive strain enhanced all tested proinflammatory factors and additionally the expression of the osmoprotective transcription factor NFAT-5. As expected, the addition of 40 mM NaCl to the cell culture medium favored the expression of NFAT-5 as well. Increased salt concentrations reduced IL-6 expression by macrophages, while TNF and *Ptgs-2*/PG-E2 expression was increased.

TNF is an endotoxin-induced macrophage-derived factor that modulates a broad spectrum of responses, including inflammation, immune regulation, proliferation, apoptosis and antiviral activity.[23] In cardiac fibroblasts TNF inhibits collagen synthesis, stimulates matrix metalloprotease synthesis[24] and increases the production of osteoclastogenic cytokines.[25,26] These results indicate that TNF stimulates osteoclast differentiation. TNF, together with IL-1β, may play an

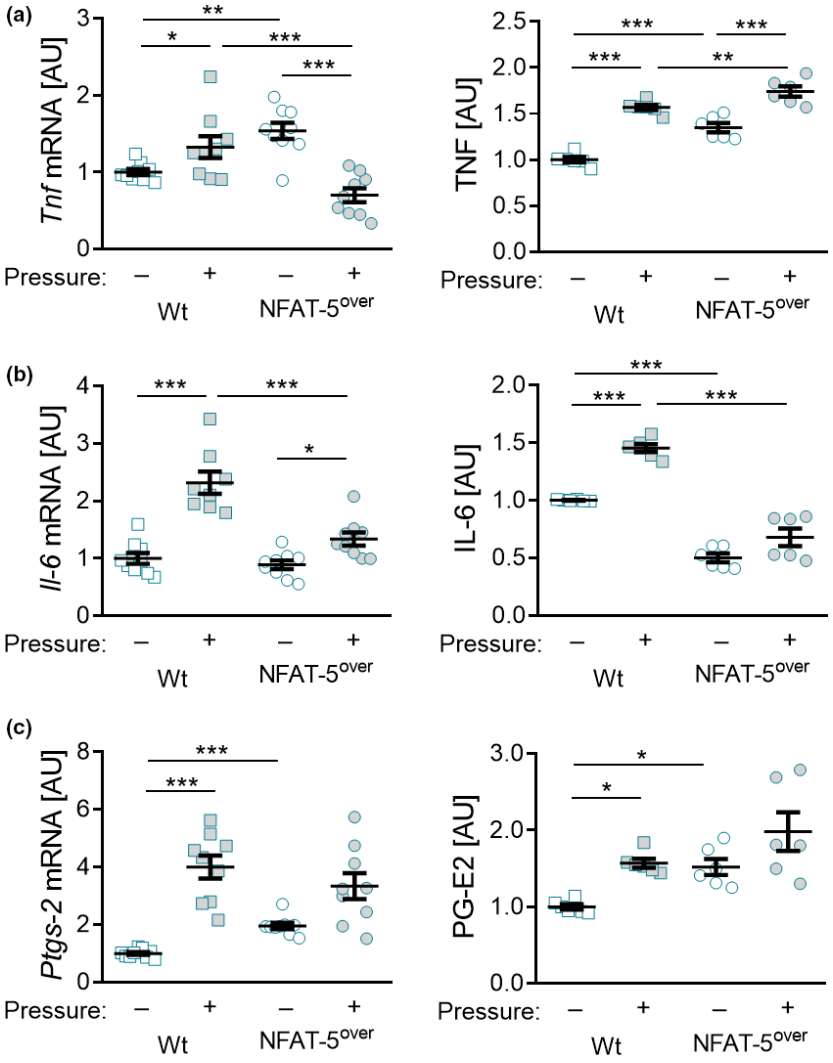

Figure 4. Gene and protein expression of **(a)** TNF, **(b)** IL-6 and **(c)** *Ptgs-2*/PG-E2 after compressive strain for 4 h in conventional RAW264.7 macrophages [wild type (Wt)] or NFAT-5-overexpressing macrophages (NFAT-5^{over}). Data were analyzed by ANOVA followed by Holm–Šídák or Games–Howell multiple comparison tests. AU, arbitrary units; IL, interleukin; mRNA, messenger RNA; NFAT-5, nuclear factor of activated T-cells-5; PG-E2, prostaglandin E2; Ptgs-2, prostaglandin endoperoxide synthase-2; TNF, tumor necrosis factor; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

important role in bone resorption of inflammatory bone diseases.[27,28] Increased TNF secretion by macrophages after pressure application may therefore enhance bone resorption in pressure zones of the periodontal ligament. As *Tnf* contains NFAT-5-binding sites,[7] we observed an increased *Tnf* expression with conditional NFAT-5 expression, indicating a possible regulatory role of NFAT-5 in *Tnf* expression.

IL-6 is a pleiotropic cytokine involved in multiple pathways, such as differentiation of monocytes, expression of matrix metalloproteinases, cell growth and metabolic processes.[29,30] In particular, IL-6 has been shown to be a powerful regulator of immune responses and inflammation.[31] It is likely that inflammation-induced IL-6 acts physiologically as a rapid downregulator of proinflammatory macrophage proliferation and as an inducer of M2 polarization to protect the organism from excessive immune responses.[32,33] In contrast to TNF, IL-6 did not stimulate the expression of RANK-L in osteoblasts.[25] Periodontal ligament fibroblasts reacted to compressive forces with a significant upregulation of IL-6 expression within 24 h.[6] Tensile strain, however, reduced IL-6 gene expression in periodontal ligament fibroblasts.[34] By contrast, macrophages reacted to pressure and tensile strain with an upregulation of IL-6 on the mRNA and protein level.

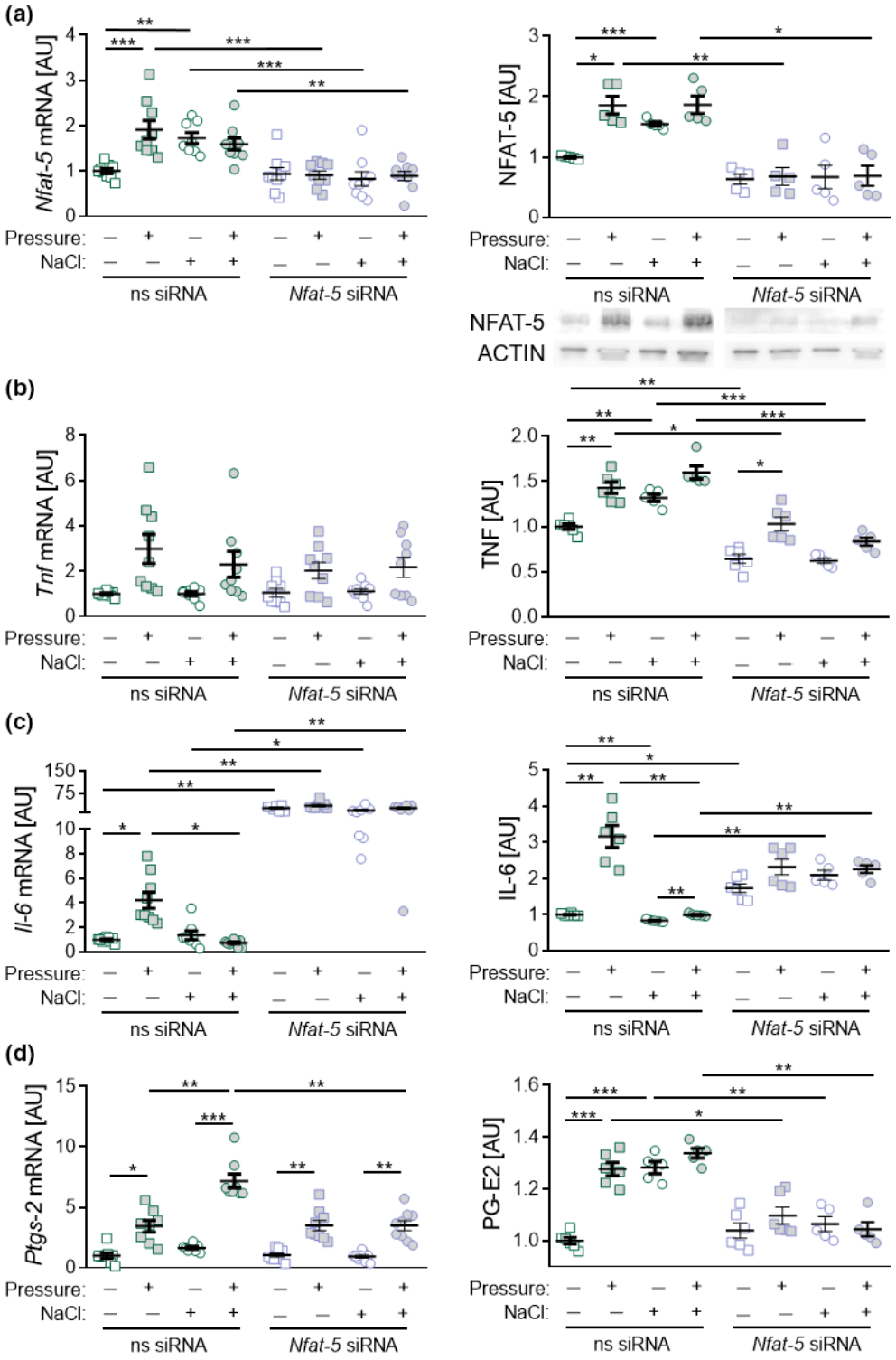

Figure 5. Gene and protein expression of **(a)** NFAT-5, **(b)** TNF, **(c)** IL-6 and **(d)** *Ptgs-2*/PG-E2 after compressive strain in nonsilencing (ns, control) or *Nfat-5*-specific siRNA-treated macrophages for 4 h. Data were analyzed by ANOVA followed by Holm–Šídák or Games–Howell multiple comparison tests. AU, arbitrary units; IL, interleukin; mRNA, messenger RNA; NFAT-5, nuclear factor of activated T-cells-5; PG-E2, prostaglandin E2; Ptgs-2, prostaglandin endoperoxide synthase-2; siRNA, small interfering RNA; TNF, tumor necrosis factor; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

The addition of NaCl, however, attenuated this effect induced by mechanical strain. As NFAT-5 overexpression resulted in reduced IL-6 expression, whereas silencing of NFAT-5 increased IL-6 significantly, NFAT-5 seems to regulate IL-6 reciprocally. The mechanisms behind this regulation, however, are still elusive.

PTGS-2 is an important enzyme in the production pathway of PG-E2 from arachidonic acid. It is known that this pathway plays a role in inflammation and tooth movement.[5,6,19] *In vitro* experiments revealed increased *Ptgs-2* expression following stimulation with proinflammatory cytokines such as IL-1 and TNF in many cell types, including synoviocytes, endothelial cells and osteoblasts.[35] PTGS-2-synthesized prostaglandins have important roles during bone resorption.[36,37] PG-E2 seems to mediate inflammatory responses and induce bone resorption by activating osteoclastic cells.[21] Mechanical strain resulted in enhanced *Ptgs-2* expression after pressure application, but not with tensile strain. As *Ptgs-2* is a known NFAT-5 target gene, we observed increased *Ptgs-2* expression in macrophages overexpressing NFAT-5, whereas *Ptgs-2* expression after NFAT-5 silencing was reduced. Enhanced *Ptgs-2* mRNA expression resulted in increased PG-E2 secretion. This enhanced PG-E2 secretion could then downstream propagate osteoclastogenesis via the RANK-L/OPG system, as already reported.[6,9]

Increased salt intake provokes and triggers chronic diseases such as hypertension or osteopenia.[14,38] A higher amount of NaCl in the culture medium of periodontal ligament fibroblasts has already been reported to result in an increased secretion of prostaglandins and expression of alkaline phosphatase and genes involved in extracellular matrix rearrangement, but decreased compression-related expression of IL-6.[19] NaCl also seems to impair the expression of proinflammatory factors via NFAT-5.

A constitutive expression of NFAT-5 reduced IL-6 expression in our experiments, whereas TNF and *Ptgs-2*/PG-E2 expressions were increased in macrophages. Inhibition of NFAT-5 upregulated IL-6 expression and reduced *Ptgs-2*/PG-E2 as well as TNF expression. The most likely explanation for this observation is that NFAT-5-binding sites exist on corresponding promotor regions, which has been reported previously for *Tnf* and *Ptgs-2*.[7,39] Interestingly, the enhancing effect of compressive strain on *Tnf* expression by macrophages was not affected by NFAT-5 inhibition in contrast to the enhancing effect of salt on *Tnf* expression, indicating that the upregulation of proinflammatory factors during compressive strain is not solely regulated by NFAT-5. The salt- and pressure-upregulated expression of the major proinflammatory factors *Ptgs-2*/PG-E2, which play an important role in the mediation of OTM in the periodontal ligament by upregulating osteoclastogenesis via the RANK-L/OPG system,[5,9,21,22] was enhanced by NFAT-5 overexpression and inhibited by NFAT-5 silencing. This indicates that NFAT-5 seems to play an important regulating role in the mediation of both compressive strain and salt effects in macrophage-mediated local inflammation in the periodontal ligament and may in consequence affect osteoclastogenesis and OTM.

Apart from macrophages, multiple different cell types are involved in the regulation of OTM at the cellular–molecular level like periodontal ligament fibroblasts and other immune cells such as leukocytes,[5,4,8,40] which might react differently to changes in local salt concentrations in the periodontal ligament. Thus, a generalization of macrophage-related effects observed in this study to OTM *in vivo* is limited and requires further investigations on these other cell types and their interaction during OTM. Previous studies investigating effects of salt (NaCl) in the context of bone metabolism and orthodontics, however, complement and concur with our results in macrophages.

In a previous study investigating the effects of salt on periodontal ligament fibroblasts in the context of OTM,[19] salt increased the release of PG-E2 as well as of RANK-L, which promotes osteoclastogenesis, by these cells already under control conditions, which would suggest complementary proinflammatory effects of salt on these cells as well as on macrophages studied here. RANK-L expression and osteoclastogenesis induced by periodontal ligament fibroblasts were significantly increased by salt exposure both under control conditions and after pressure application. Macrophages may in interaction with these cells have a potentiating effect under high salt conditions *in vivo*, which merits further investigation. In a previous *in vivo* study on mice, we observed a lower bone density in the tibia after a 2-week high-salt diet compared with controls on a low-salt diet.[41] This reduction in bone density was caused by an increased number of osteoclasts, whereas the number of osteoblasts did not change with the diet. This indicates that an increased salt consumption could possibly promote osteoclast differentiation in the periodontal ligament, which may entail an acceleration of OTM. However, periodontal bone loss and tooth root resorption as detrimental side effects may also be possible effects of salt consumption *in vivo*, as these can also be induced by increased and uncontrolled osteoclast activity. Based on the available evidence from this and previous studies, we can surmise that both periodontal ligament fibroblasts and macrophages contributed to this salt-induced effect.

We conclude from our results that salt (NaCl) has an influence on the gene and protein expression of the proinflammatory factors TNF, IL-6 and *Ptgs-2*/PG-E2 and influences the expression profile of macrophages as a reaction to mechanical stimuli (i.e. compressive and tensile strain) that occur during OTM. As a result, sodium retention in the periodontal ligament caused by dietary salt consumption and a resulting upregulation of inflammatory processes could possibly propagate local osteoclastogenesis and thus increase the velocity of OTM, and this may also lead to dental root resorptions or periodontal bone loss, which needs to be investigated further.

METHODS

Cell culture conditions, macrophage cell types and siRNA transfection

RAW264.7 macrophages (400319, CLS Cell Lines Service, Eppelheim, Germany, NFAT-5 wild type) and NFAT-5-overexpressing macrophages (NFAT-5^{over14}) were investigated. Furthermore, we silenced NFAT-5 (NFAT-5 siRNA) in RAW264.7 macrophages (L058868; Dharmacon, Lafayette, LA, USA) and compared them with RAW264.7 macrophages treated with nonsilencing siRNA (1027281, Qiagen, Hilden, Germany). All cells were cultured in Dulbecco's modified Eagle's medium [high glucose (4.5 g L^{-1}); D5671; Sigma-Aldrich, St. Louis, MO, USA] with 10% fetal bovine serum (P30-3302; PAN-Biotech, Aidenbach, Germany), 1% antibiotic/antimycotic solution (A5955; Sigma-Aldrich) and 1% L-glutamine (G7513; Sigma-Aldrich). All media, solutions and buffers were processed under sterile conditions and preheated to 37°C before use. We transferred siRNA as already described previously.[42] Briefly, we put the siRNA into a 4-mm cuvette (Molecular Bioproducts, San Diego, CA, USA) and added OPTI-MEM (11058021; Thermo Fisher Scientific, Waltham, MA, USA) to a total volume of 50 µL. Next we scraped the macrophages off the cell culture flask, determined cell number using a Neubauer counting chamber and added 50 µL of the cell suspension containing 2×10^6 RAW264.7 macrophages in OPTI-MEM and pulsed them in a Gene Pulser Xcell (Bio-Rad, Hercules, USA). Pulse conditions were 400 V, 150 µF, 100 Ω.

Experimental setup for *in vitro* experiments

Approximately 250 000 macrophages per mL were cultured either on 6-well BioFlex plates (BF-3001U; Flexcell International Corporation) for tensile strain or on conventional polystyrol 6-well plates (83.3920; SARSTEDT, Nürnbrecht, Germany) for pressure application under cell culture conditions. Cells either remained untreated for 4 h (control) or were subjected to tensile strain (16%, isotropic stretching) or compressive strain (2 g cm^{-2}) with or without addition of 40 mM NaCl (high/normal salt conditions). The incubation time of 4 h proved to be sufficient to observe effects on the expression of investigated cytokines.[43] Tensile strain was achieved using custom-made silicon stamps, which led to an isotropic stretching of about 16% (Supplementary figure 2).[34] Compressive force treatment was performed using a glass plate of a distinct weight, which is widely used to simulate the forces occurring in pressure zones of the periodontal ligament during OTM (Supplementary figure 2).[6,9] In addition, we performed experiments with 24 h of mechanical loading (Supplementary figures 3, 4). At the end of the incubation period, we examined gene expression by real-time-quantitative PCR and protein expression by ELISA and Western blots.

RNA extraction

Macrophages were scraped off in ice-cold phosphate-buffered saline, centrifuged at 4°C and 2556 *g* for 10 min (HERAEUS Fresco 17 Centrifuge; Thermo Fisher Scientific) and the supernatant was discarded to obtain a cell pellet, which was taken up in 500 µL of peqGOLD TriFast (30-2010; VWR International, Radnor, PA, USA). Subsequently, 100 µL of chloroform (1.02445.1000, VWR International) was added and vortexed for 30 s. After 15 min on ice, samples were centrifuged at 4°C and 16,200 *g* for 15 min. The aqueous supernatant was transferred to a cup containing 500 µL isopropanol (20842,330, VWR International) and incubated at −80°C overnight. Samples were centrifuged at 4°C for 30 min, the supernatant was removed, 500 µL of 80% ethanol (32205, Sigma-Aldrich, St. Louis, USA) was added to wash the pellet and samples were centrifuged again for 10 min. This process was repeated. Finally, the pellet was dried for at least 30 min. To determine RNA concentration, the pellet was taken up in 20 µL of RNase-free H_2O (T143; Carl Roth, Karlsruhe, Germany) and measured at 260 nm in a photometer (Implen, Munich, Germany).

Complementary DNA synthesis

Reverse transcription reaction was performed in a total volume of 10 µL mixture consisting of 5.5 µL of RNA and 4.5 µL of master mix containing 2 µL of Moloney murine leukemia virus buffer (M531A; Promega, Madison, WI, USA), 0.5 µL Oligo(dT) (SO132; Thermo Fisher Scientific), 0.5 µL Random Hexamer (SO142; Thermo Fisher Scientific), 0.5 µL deoxynucleoside triphosphates (L785.1; Carl Roth), 0.5 µL RNase Inhibitor (EO0381; Thermo Fisher Scientific) and 0.5 µL Moloney murine leukemia virus reverse transcriptase (M170B; Promega). The samples were incubated at 37°C for 1 h and 95°C for 2 min. Samples were diluted 1:5 by addition of RNase-free H_2O (T143; Carl Roth) and stored at −20°C until use.

Quantitative real-time polymerase chain reaction

For real-time-quantitative PCR analysis 1.5 µL of diluted complementary DNA was pipetted onto a 96-well plate, briefly centrifuged and 13.5 µL primer mix consisting of 0.375 µL forward primer, 0.375 µL reverse primer, 7.5 µL SYBR Green

Table 1. Reference genes (*Eef1a1/Sdha*) and target genes for real-time-quantitative PCR.

Gene symbol	Gene name	Accession number	5′-Forward primer-3′	5′-Reverse primer-3′
Eef1a1	Eukaryotic translation elongation factor 1 alpha 1	NM_010106.2	AAAACATGATTACAGGCACATCCC	GCCCGTTCTTGGAGATACCAG
Sdha	Succinate dehydrogenase complex, subunit A	NM_023281.1	AACACTGGAGGAAGCACACC	AGTAGGAGCGGATAGCAGGAG
Ptgs-2	Prostaglandin endoperoxide synthase-2	NM_011198.4	TCCCTGAAGCCGTACACATC	TCCCCAAAGATAGCATCTGGAC
Il-6	Interleukin-6	NM_031168.2	ACAAAGCCAGAGTCCTTCAGAG	GAGCATTGGAAATTGGGGTAGG
Nfat5	Nuclear factor for activated T cells 5	NM_133957.3	AAATGACCTGTAGTTCTCTGCTTC	GCTGTCGGTGACTGAGGTAG
Tnf	Tumor necrosis factor	NM_013693.3	TCGAGTGACAAGCCTGTAGCC	CTTTGAGATCCATGCCGTTGGC

JumpStart (S4438, Sigma-Aldrich) and 5.25 µL RNase-free H_2O (T143, Carl Roth) was added. After centrifugation, the plate was covered with an adhesive optical film (712350; Biozym, Hessisch Oldendorf, Germany) and placed in a thermal cycler (RealPlex2; Eppendorf, Hamburg, Germany). After 95°C for 5 min, amplification was performed in 45 cycles with 10 s at 95°C, 8 s at 60°C, 8 s at 72°C followed by a melting curve analysis.

To normalize target gene expression, reference genes *Eef1a1* and *Sdha* were used (Table 1), which proved to be stably expressed under the experimental conditions tested (data not shown). C_q values were defined as the second derivative maximum of the fluorescence signal curve at 521 nm and calculated with the realplex software (version 2.2; Eppendorf AG, CalQPlex algorithm). Relative gene expression was calculated with the formula $2^{-\Delta Cq}$,[44,45] with $\Delta C_q = C_q$ (target gene) $- C_q$ (mean *Eef1a1/Sdha*). All primers (Table 1) were prepared and real-time-quantitative PCR quality control was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments quality guidelines as described previously.[47–49] Unmodified primers were synthesized and purified by Eurofins MWG Operon LLC (Huntsville, AL, USA). For each primer pair and each quantitative PCR, a no-template-control without complementary DNA was analyzed.

Enzyme-linked immunosorbent assays

Cell culture supernatants were stored at −80°C until use and thawed on ice. ELISAs were performed according to the manufacturer's instructions (PG-E2: MBS266212; MyBioSource, San Diego, CA, USA; IL-6: MBS335514; MyBioSource; TNF-α: MBS335449; MyBioSource). Fold changes were calculated from concentrations relative to cell number, which are presented as Supplementary figures 5 and 6.

Western blot analysis

Protein purification was carried out with a solution of 8 M urea (U5378; Sigma-Aldrich, Munich, Germany) supplemented with a proteinase inhibitor mix (87786; Thermo Fisher Scientific). Protein concentration was determined with a Bradford test (K015.3; Carl Roth) and same amounts of protein were used for Western blot analyses. Polyacrylamide gel electrophoresis was performed with the Bio-Rad apparatus (Mini-PROTEAN Tetra Vertical Electrophoresis Cell, 1658004; Bio-Rad, Hercules, CA, USA). Because of the protein size of more than 100 kDa, 8% separating gels were used. Proteins were transferred from gel to a polyvinylidene fluoride membrane, T830.1; Carl Roth). After blocking the membrane in 5% milk (T145.3; Carl Roth) in TBS-T (10× Tris-buffered saline: 24.23 g Trizma base; T1503; Sigma-Aldrich), 80 g NaCl (3957.1; Carl Roth) and H_2O_d to 1 L, 1× TBS-T + 0.1% Tween-20 (9127.1; Carl Roth) for 1 h at room temperature, membranes were incubated overnight with a primary antibody for NFAT-5 (PA1-023; Thermo Fisher Scientific; diluted 1:1000) or ACTIN (E1C602; EnoGene, New York, NY, USA; diluted 1:3000). The membranes were washed three times with TBS-T for 5 min and then incubated for an additional hour with horseradish peroxidase-conjugated antirabbit IgG (611-1302, Rockland Immunochemicals, Gilbertsville, PA, USA) diluted 1:5000 in 5% milk in TBS-T at room temperature. The membranes were washed again three times with TBS-T at room temperature for 5 min. Subsequently, the membranes were coated with Luminata Crescendo Western HRP substrates (WBLUR0100; Sigma-Aldrich, St. Louis, Germany) and recording was performed using the VWR GenoPlex documentation system (VWR International). Densitometric evaluation was carried out with ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA). Uncropped images of Western Blots are presented as supplementary information.

Statistical analysis

Prior to statistical analysis, all absolute data values were divided by the respective arithmetic mean of the control group without mechanical strain to obtain normalized data values relative to these controls, set to 1. Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). In figures, symbols represent single data points,

horizontal lines the arithmetic mean and vertical lines the standard error of the mean. All data were tested for normal distribution (Shapiro–Wilk test). Normally distributed data sets were independently compared by ordinary one-way ANOVA followed by Holm–Šídák multiple comparison tests, whereas the remaining data sets were compared by Welch-corrected ANOVAs followed by Games–Howell multiple comparison tests for heterogeneous variances. All differences were considered statistically significant at $P \leq 0.05$.

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AUTHOR CONTRIBUTION

Agnes Schröder: Conceptualization; Supervision; Visualization; Writing-original draft. **Alexandra Leikam:** Investigation. **Paul Käppler:** Investigation. **Patrick Neubert:** Methodology; Supervision. **Jonathan Jantsch:** Methodology; Supervision. **Wolfgang Neuhofer:** Resources. **James Deschner:** Supervision; Writing-review & editing. **Peter Proff:** Project administration; Writing-review & editing. **Christian Kirschneck:** Data curation; Methodology; Project administration; Supervision; Writing-original draft; Writing-review & editing.

CONFLICT OF INTEREST

The authors report no financial, commercial or other conflict of interest relevant to this article, which is the intellectual property of the authors.

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SUPPORTING INFORMATION

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