Molecular Nutrition & Food Research
www.mnf-journal.com

[6]-Gingerol Facilitates CXCL8 Secretion and ROS Production in Primary Human Neutrophils by Targeting the TRPV1 Channel

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Scope: Clarifying the function of sensory active TRP (transient receptor potential) channels in non-sensory tissue is of growing interest, especially with regard to food ingredients in nutritionally relevant concentrations. The study hypothesizes the TRPV1 agonist [6]-gingerol to facilitate cellular immune responses of primary human neutrophils, after treatment with 50 nM, a concentration that can be reached in the circulation after habitual dietary intake.

Methods and results: qRT-PCR analyses reveal a high abundancy of TRP channel RNA expression in the types of primary leukocytes investigated, namely neutrophils, monocytes, NK cells, T cells, and B cells. Incubation of neutrophils with 50 nM of the known TRPV1 ligand [6]-gingerol led to increased surface expression of CD11b, CD66b, and the fMLF receptor FPR1, as shown by flow cytometry. Upon subsequent stimulation with fMLF, the neutrophils display an about 30% ($p < 0.05$) increase in CXCL8 secretion as well as in ROS production. Pharmacological inhibition of TRPV1 by *trans-tert*-butylcyclohexanol abolishes the [6]-gingerol induced effects.

Conclusions: The TRPV1 channel is functionally expressed in human neutrophils. Activation of the channel with [6]-gingerol as a food-derived ligand in nutritionally relevant concentrations leads to an enhanced responsiveness in the cells towards activating stimuli, thereby facilitating a canonical cellular immune response in human neutrophils.

1. Introduction

Identifying food ingredients with immunomodulatory properties together with their respective molecular targets has attracted increased interest in recent years. Due to their high ligand diversity, the transient receptor potential superfamily of ion channels (TRP channels) represents a very interesting class of potential target structures. The mammalian TRP channel superfamily includes six related protein families, namely the ankyrin (TRPA), the canonical (TRPC), the melastatin (TRPM), the mucolipin (TRPML), the polycystin (TRPP), and the vanilloid (TRPV) family, all of which typically share six transmembrane segments that assemble as tetramers to form cation-permeable pores with varying cation selectivity.[1] The TRPV1 channel may be, by far, the most intensively investigated member of the TRP superfamily. TRPV1 was initially identified as the receptor for capsaicin, the pungent component of chili peppers.[2] Later, also the pungent ingredients from ginger, the gingerols, were shown to activate TRPV1.[3] The biological role of TRPV1 in non-neuronal cell types is still under extensive investigation, yet the results available imply functions far beyond sensory and thermal perception. For example, a 12-week intervention with a daily dose of 0.15 mg of the TRPV1 agonist nonivamide, a structural capsaicin analogue, prevented a dietary-induced gain in body fat mass, and increased plasma serotonin levels in healthy overweight subjects.[4] In 3T3-L1 adipocytes activation of TRPV1 by nonivamide decreased lipid accumulation during differentiation and maturation by suppressing PPARγ expression.[5] In macrophages, capsaicin and nonivamide attenuated an LPS-induced release of pro-inflammatory cytokines like IL6, CXCL8, and TNF-alpha, in a TRPV1-dependent manner.[6] Yet, the particular functions of TRPV1 in human blood leukocytes remain vague. In human NK cells, 10 and 50 µM capsaicin induced a rise in intracellular Ca^{2+} concentrations, indicating a functional TRPV1 channel.[7] However, dampened NK cell effector functions such as cytotoxic degranulation and cytokine secretion, induced by pre-treatment of the cells with capsaicin for 1 h in a concentration range of 10–100 µM, were largely TRPV1 independent.[7] In T cells, TRPV1 was documented to be involved in the processes of T cell

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202200434

DOI: 10.1002/mnfr.202200434

receptor signaling, T cell proliferation and differentiation, as well as cytokine production.[8] Previous work from our group also demonstrated functional expression of TRPV1 in human primary T cells.[9] Furthermore, dose response analyses in concentrations ranging from 0.03 to 300 μmol L^{-1} revealed that [6]-gingerol inhibits cytokine secretion by primary human leukocytes with an IC_{50} value of 82.2 μmol L^{-1}. However, quantitation of [6]-gingerol in plasma samples of healthy subjects revealed a mean maximum plasma concentration of only 42.0 ± 16.3 nmol L^{-1} after the intake of 1 L of ginger tea. Since these [6]-gingerol concentrations had no significant impact on cytokine secretion in previous studies,[9] it is unclear, whether a dietary relevant concentration of 50 nmol L^{-1}, being reached in blood plasma after consumption of 1 L of ginger tea, is sufficient to modulate cellular immune responses in other human primary leukocytes.

For human neutrophils, knowledge about the functional role of TRPV1 is limited. Whereas Köse and Nazıroğlu[10] showed Ca^{2+}-fluxes in neutrophils in response to 10 μM capsaicin to be decreased by the TRPV1 antagonist capsazepine, other results did not demonstrate capsaicin to induce a Ca^{2+} influx when tested in a concentration range of 1–100 μM, despite a detectable TRPV1 RNA expression.[11]

Neutrophils are the most abundant leukocytes in human blood, accounting for 60–70 % of all circulating white blood cells. They are the first immune cells that are recruited to the sites of infection; they are therefore often referred to as the first line of defense.[12] Recruitment of neutrophils is triggered by, among others, the bacterial or mitochondria-derived peptide N-Formylmethionine-leucyl-phenylalanine (fMLF) or chemokines such as CXCL8 (IL-8).[13] Defense mechanisms of neutrophils include phagocytosis,[14] anti-microbial enzyme release via degranulation,[15] generation of reactive oxygen species (ROS),[16] and the formation of neutrophil extracellular traps.[17] Besides these direct defense mechanisms, neutrophils further contribute to subsequent immune responses via the release of various cytokines and chemokines.[18] Also, neutrophils can undergo a priming process that enables them to respond more strongly to subsequent full activation.[16b]

In recent years, evidence has grown that ingredients from food and or medicinal plants can modify one or more of the mentioned defense responses of human neutrophils. These modifications include increased phagocytotic activity[19] and ROS generation,[20] augmented chemotaxis towards fMLF,[21] and formation of neutrophil extracellular traps.[20] However, the active compound(s) were not identified. Constituents from *Ferula akitschkensis* (*β*-pinene, sabinene, *γ*-terpinene, geranylacetone, and isobornylacetate) desensitized neutrophils to fMLF- and CXCL8-induced Ca^{2+} influx and inhibited fMLF induced chemotaxis, wherein the geranylacetone-induced effects were mediated via TRPV1.[22]

Based on the available data, we hypothesized that ligand induced activation of TRPV1 by [6]-gingerol can affect general neutrophil functions, either directly or via enhancing their responses to activating stimuli. Within the scope of this hypothesis, we particularly aimed at elucidating, whether a verified nutritionally relevant concentration is sufficient to modulate cellular immune responses in human primary neutrophils as part of the leukocyte population. Furthermore, we sought to compare the RNA expression levels of all members of the mammalian TRP superfamily in five of the most prominent cell types in human blood in order to obtain a qualitatively and quantitatively comprehensive overview of TRP channel expression in human leukocytes.

2. Results

2.1. Abundance and Relative Transcript Levels of TRP Channels in Human Leukocytes

In order to evaluate TRP channel RNA expression in blood leukocytes, five of the most prominent leukocyte cell types were isolated from the blood of healthy donors and the RNA expression of TRP channels was analyzed via quantitative RT-PCR (**Figure 1**).

Specific transcripts of the TRPV as well as the TRPM family were detected with high frequencies of 75–100% in all cell types analyzed. The mean overall frequency considering all cell types was 96% for the TRPV family and 98% for the TRPM family. TRPC-specific transcripts were much less abundant, ranging from 0% in monocytes, NK cells, T cells, and B cells to 100% in NK cells, and T cells with an overall mean frequency of only 53% (Figure 1A). The TRPC5-specific transcript was only detected in neutrophils, with a frequency of 70%. Also, the TRPA1-specific transcript generally revealed a rather low abundancy in the cell types analyzed with a frequency of 100% in neutrophils, 25% in monocytes, 80% in NK cells, 100% in T cells, and 60% in B cells. Likewise, the TRPML3-specific transcript showed a low frequency in neutrophils (50%), monocytes (25%), NK cells (40%), and B cells (20%), but a frequency of 100% in T cells. The TRPV1-specific transcript was detected in all cell types, showing a frequency of 100% in monocytes, NK cells, and T cells, and a frequency of 90% in neutrophils and 80% in B cells.

Regarding relative RNA expression, as compared to the respective frequencies, the TRP channels revealed a more cell type-specific expression pattern, as evident by comparison of the respective Δct values in the different cell types analyzed (Figure 1B). For example, TRPP3 was detected with a frequency of 100% in all cell types, but clearly revealed the highest expression level in monocytes. In contrast, the TRPV2 channel was detected in all of the cell types investigated, with a comparably high RNA-expression level as well as a high frequency. Likewise, transcript levels of TRPV1 were similar in all cell types examined (Figure 1B).

2.2. TRPV1 Surface Expression on Neutrophils

The current knowledge about the function of TRPV1 in human neutrophils is still unclear. To further explore the roles of TRPV1 in human neutrophils, we next investigated whether the TRPV1 channel is expressed on the surface of primary human neutrophils using live cell flow cytometry (**Figure 2**). The isolated neutrophils were stained for CD15 as a surface marker for neutrophils (Figure 2A,B) and simultaneously either stained with an antibody raised against an epitope in the first extracellular loop of the TRPV1 protein (Figure 2B) or the respective isotype control, the latter serving as a surrogate for measuring unspecific binding (Figure 2A). Within the $CD15^+$ population, the fluorescence intensity for FITC was analyzed. Staining of the neutrophils with

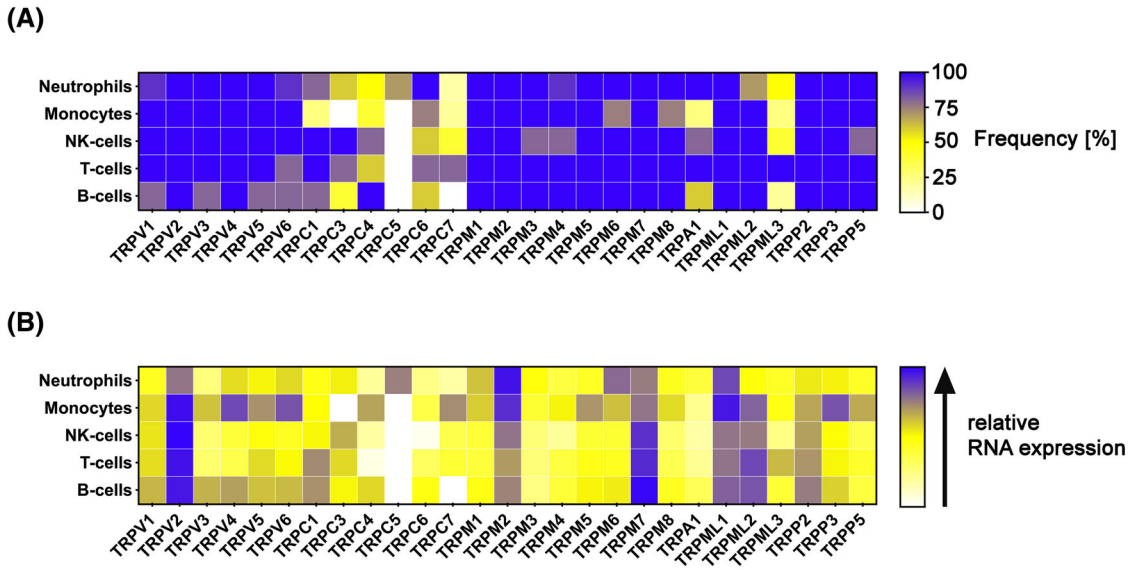

Figure 1. Human leukocytes express TRP-specific transcripts. A) Qualitative PCR analysis of transcripts encoding TRP proteins in primary human blood leukocytes. B) Quantitative PCR analysis of specific transcripts encoding TRP proteins in primary human blood leukocytes. Data are presented as heatmaps resulting from the q-RT-PCR analyses of up to 10 individual donors.

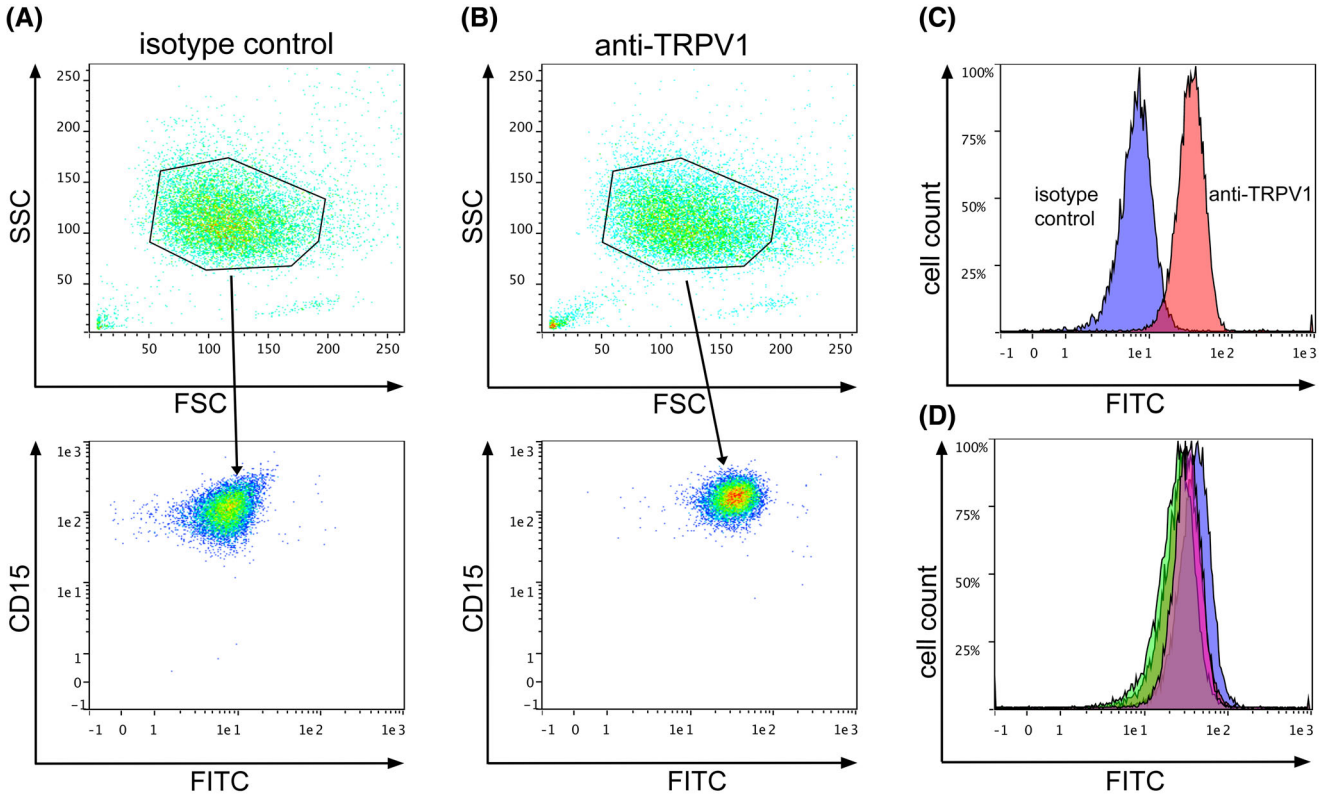

Figure 2. The TRPV1 channel is expressed on the surface of human neutrophils. Flow cytometry of isolated human neutrophils, stained either with a TRPV1 specific antibody B) or the corresponding isotype control A), both conjugated to FITC. Cells were additionally stained for CD15. Numbers in the plots indicate either values for forward and side scatter or mean fluorescence intensity of the staining. C) Histogram overlay of the stains for TRPV1 and isotype control. D) Histogram overlay of the stains for TRPV1 of four individual donors.

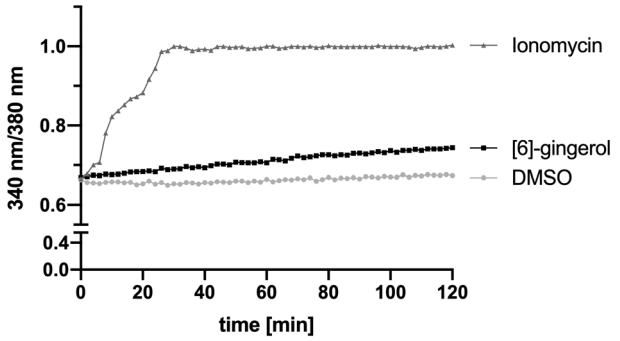

Figure 3. Incubation of neutrophils with [6]-gingerol leads to increased intracellular Ca^{2+} concentrations. Isolated human neutrophils were loaded with Fura-2 AM, and the fluorescence intensity assessed after the addition of either 1 µM Ionomycin, 50 nM [6]-gingerol, or 0.02% DMSO as solvent control. Data are representative of four independent experiments.

the TRPV1 antibody led to a clearly distinguishable fluorescence signal compared to the isotype control, thereby confirming surface expression of TRPV1 in primary human neutrophils (Figure 2C). Analyzing neutrophils from four individual donors revealed a comparable surface expression of TRPV1 (Figure 2D).

2.3. [6]-Gingerol Induced Increase in Intracellular Ca^{2+}

Since ligand-induced activation of TRPV1 will result in an influx of Ca^{2+},[23] intracellular Ca^{2+} concentrations of neutrophils were determined via the Ca^{2+}-sensitive dye Fura-2. Based on our previous findings,[9] a concentration of 50 nM of the well-known TRPV1 ligand [6]-gingerol and an incubation time of 2 h were chosen. The analyses showed that incubation of neutrophils with 50 nM [6]-gingerol resulted in increased intracellular Ca^{2+} concentrations which were on average 18.4% ± 1.0% of the maximum value, as determined by applying 1 µM of the ionophore ionomycin. The increase induced by DMSO was at 4.7% ± 1.2% (**Figure 3**).

2.4. Impact of [6]-Gingerol on TRPV1 Expression

Next, we aimed at analyzing the impact of TRPV1 stimulation by [6]-gingerol on TRPV1 expression at the transcript level via q-RT-PCR as well as at the surface protein level via live cell staining. For this purpose, human neutrophils were incubated with 50 nM [6]-gingerol for 2 h and the respective expression levels quantified.

This 2 h incubation impacted neither TRPV1 transcript nor protein levels (**Figure 4**), and also did not change, except for IL6, IL17A, IL24, C5, and GDF5, the RNA expression of common cytokine and chemokine genes investigated (Figure S1, Table S2, Supporting Information).

2.5. Impact of [6]-Gingerol on Expression of Neutrophil Surface Markers

In this set of experiments, we asked whether [6]-gingerol has an impact on the expression of common surface markers for neutrophils. We found that the expression of the markers CD11b and

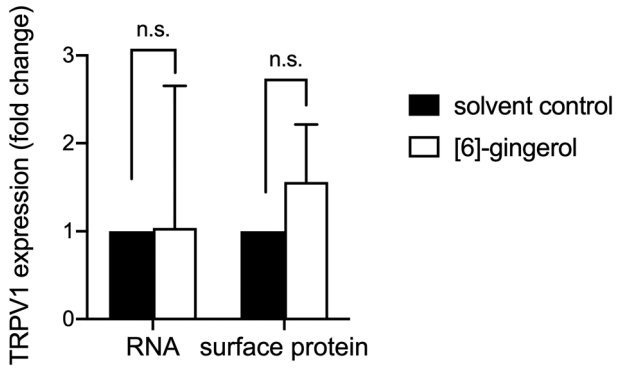

Figure 4. Incubation of human neutrophils with [6]-gingerol does not influence TRPV1 expression. Isolated human neutrophils were incubated with 50 nM [6]-gingerol or 0.02% DMSO as solvent control for 2 h. Afterwards, TRPV1 expression was either determined via q-RT-PCR for RNA expression, or via flow cytometry for protein surface expression. Data are shown as mean ± SD from four independent experiments, respectively. Statistical differences were calculated by Student's *t*-test (two-tailed, paired). n.s. = $p > 0.05$.

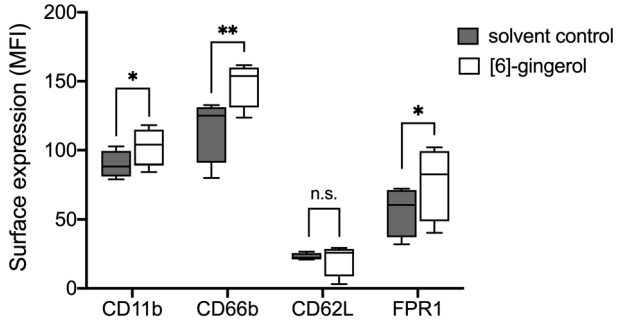

Figure 5. Incubation of human neutrophils with [6]-gingerol impacts surface expression of neutrophil activation markers. Isolated human neutrophils were stained for CD11b, CD66b, CD62L, and FPR1 after incubation of the cells with 50 nM of [6]-gingerol or 0.02% DMSO as solvent control and analyzed using flow cytometry. Staining for CD15 served as identification marker. Data are shown as box and whiskers (min to max) of mean fluorescence intensity (MFI), derived from four independent experiments in which solvent-treated control samples (control) were compared with samples treated with [6]-gingerol. Statistical differences were calculated by Student's *t*-test (two-tailed, paired). n.s.: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$.

CD66b was increased after the 2 h incubation period with [6]-gingerol when compared to the solvent control, whereas CD62L was unaffected (**Figure 5**). Furthermore, surface expression of the fMLF receptor FPR1 was significantly elevated after treatment of the cells with 50 nM [6]-gingerol as compared to the solvent control.

2.6. Impact of [6]-Gingerol on CXCL8 Secretion upon fMLF Stimulation

The previous result prompted the question whether the altered protein expression on the surface of neutrophils affects general responses of these cells to well-known activating molecules like fMLF. When isolated neutrophils were pre-incubated with [6]-gingerol in a concentration of 50 nM for 2 h and stimulated with

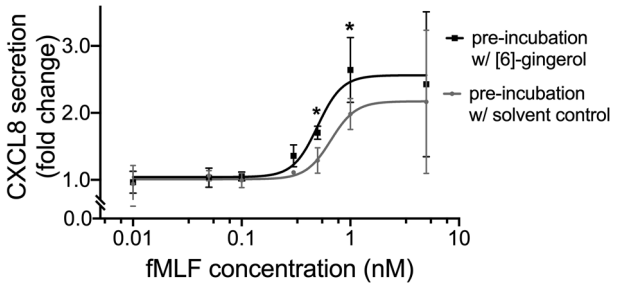

Figure 6. Pre-incubation with [6]-gingerol increases CXCL8 secretion of human neutrophils upon stimulation with fMLF. Isolated human neutrophils were incubated either with 50 nM [6]-gingerol or 0.02% DMSO as solvent control for 2 h. Afterwards, the solutions were washed out and the cells were incubated with fMLF at the concentrations indicated for 4 h. CXCL8 in the respective supernatants was quantified via ELISA. Data are shown as mean ± SD of four independent experiments. Statistical differences were calculated by Student's *t*-test (two-tailed, paired). *: $p \leq 0.05$, **: $p \leq 0.01$.

different concentrations of fMLF for additional 4 h, an increase in CXCL8 secretion after subsequent stimulation with fMLF at concentrations of 0.3 nM (22%, $p = 0.08$), 0.5 nM (32%, $p = 0.05$), and 1 nM (33%, $p = 0.03$) was demonstrated as compared to sole stimulation with fMLF (**Figure 6**). Also, pre-incubating the neutrophils for 2 h with 50 nM [6]-gingerol promoted a shift of the EC_{50} value from 0.66 to 0.49, indicating an augmented responsiveness of the cells towards fMLF. In contrast, pre-incubating the neutrophils with 50 nM [6]-gingerol for 2 h alone did not induce CXCL8 secretion (CXCL8 not detectable, data not shown).

2.7. Impact of [6]-Gingerol on ROS Production

Next, we analyzed whether incubation of neutrophils with [6]-gingerol affects their generation of reactive oxygen species after the 2 h incubation period with [6]-gingerol and after subsequent stimulation with 1 nM fMLF.

The analyses showed no statistically significant impact of a 2 h incubation of neutrophils with 50 nM [6]-gingerol (**Figure 7**A). However, pre-incubating neutrophils for 2 h with 50 nM of [6]-gingerol and subsequently stimulating the cells with 1 nM fMLF did increase the ROS production by 27.8% ± 2.2% (Figure 7B).

2.8. Pharmacological Inhibition of TRPV1 Reverses [6]-Gingerol-Induced Effects

In order to verify the involvement of TRPV1 in the [6]-gingerol-induced increases in CXCL8 secretion and ROS production after fMLF stimulation, the TRPV1 specific inhibitor *trans-tert*-butylcyclohexanol (BCH) was applied.[5a] Binding of BCH to TRPV1 interrupts its interaction with [6]-gingerol, and should thereby inhibit the [6]-gingerol-induced effects, which would allow the conclusion that the observed effects are TRPV1 mediated. For this approach, human neutrophils were either incubated with [6]-gingerol for 2 h or with [6]-gingerol and BCH for 2 h, and subsequently stimulated with 1 nM fMLF. We could show, that the increase in CXCL8 production induced by pre-incubating the neutrophils with 50 nM [6]-gingerol and subsequent stimulation with 1 nM fMLF could not be detected, when the cells were pre-incubated with a combination of [6]-gingerol and 100 µM BCH (**Figure 8**A), confirming the involvement of TRPV1 in the [6]-gingerol induced effects. In the case of ROS production, pre-incubating the neutrophils with a combination of [6]-gingerol and BCH led to even lower values than the solvent control (Figure 8B).

3. Discussion

While the chemosensory properties of some TRP channels have been well described, their function in non-sensory tissues is far less clear. Regarding their general expression profile in non-sensory tissue like blood leukocytes, only a few studies have been carried out in the past. In 2011, Wenning et al.[24] analyzed the RNA expression of members of the TRPC, TRPM, and TRPV families in primary human $CD4^+$ T cells via RT-PCR. They found consistent expression of TRPC1, TRPC3, TRPV1, TRPM2, and TRPM7. Furthermore, TRPC3 was shown to modulate the Ca^{2+}-dependent proliferation of primary $CD4^+$ T cells. Regarding the expression profile, the reported results[24] are consistent with our data, except for TRPC3, which, in the present work, was detected only with a frequency of 80%. In human neutrophils, Heiner et al.[11] analyzed the expression pattern of TRP-specific transcripts via RT-PCR. They demonstrated specific transcripts for LTRPC2 (TRPM2), TRPV1, vanilloid receptor-like protein 1 (TRPV2), epithelial Ca^{2+} channel 1 (TRPV5), epithelial Ca^{2+} channel 2 (TRPV6), and TRPC6, which is completely in line with our results.

However, until now, according to our knowledge, no comprehensive quantitative data about the RNA expression of all 27 members of the human TRP superfamily have been analyzed for five of the most prominent blood leukocyte cell types. The results we obtained allow us to draw the conclusion that the TRP channel expression is not lineage-dependent. Hence, one might hypothesize that expression of TRP channels is regulated rather individually. Which circumstances influence the expression of each TRP channel in each cell type at a certain point in time can only be speculated, but might, besides general physiological aspects, also include the individual's dietary habits and the nutritional status. For example, it was shown that high glucose levels increased the expression of TRPC1, TRPC3, TRPC5, TRPC6, TRPM6, and TRPM7 in human monocytes.[25] It is noteworthy that we could not detect TRPC3 and TRPC5 at all in monocytes, which could be explained by analyzing cells from different donors, supporting a highly individual regulation of TRP channel expression. On the other hand, stimulation of neutrophils with the TRPV1 ligand [6]-gingerol at a concentration of 50 nM for 2 h showed no significant impact on its RNA and surface protein expression. The latter result may hint at the conclusion that the incubation does not lead to an internalization of the receptor. This is in accordance with the finding that capsaicin provokes TRPV1 internalization only at higher concentrations (EC_{50}: 500 nM) in $TRPV1^+$ HEK293 cells.[26]

Incubating neutrophils with [6]-gingerol in a concentration of 50 nM for 2 h led to significant changes in protein surface expression other than TRPV1, i.e., an increased expression of the fMLF receptor FPR1, CD11b, and CD66b. This finding was interesting in that it has previously been shown that priming of neutrophils

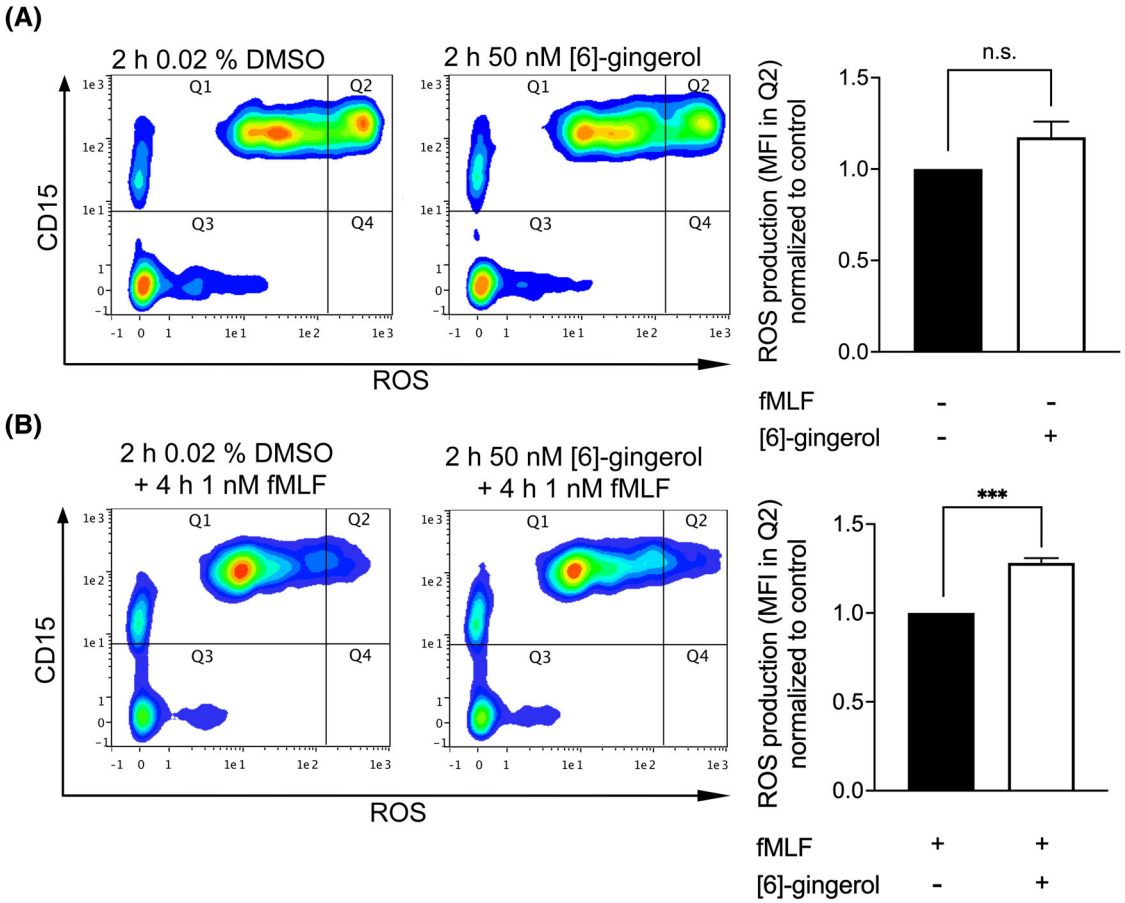

Figure 7. Pre-incubation of human neutrophils with [6]-gingerol facilitates ROS production upon fMLF stimulation. Isolated human neutrophils were stained for ROS after 2 h of incubation with 0.02% DMSO as solvent control or 50 nM of [6]-gingerol A) and after subsequent incubation with 1 nM fMLF B). Numbers in the plots indicate the mean fluorescence intensity of the staining. CD15 was used as an identification marker. The data represent three independent experiments and are given as mean ± SD. Statistical differences were calculated by Student's *t*-test (two-tailed, paired). n.s.: $p > 0.05$, ***: $p \leq 0.001$.

results in elevated surface expression of the fMLF receptor FPR1, CD11b, CD35, and CD66b.[27] Inversely, CD62L expression was reduced via enzymatic shedding in primed neutrophils.[28] Priming is a process by which neutrophils are transferred from a resting state to a "ready to go" state that is characterized by an ability to respond more strongly to activating stimuli, without features of full activation, e.g., ROS production.[16b] Whether the observed phenotypic changes can be attributed to neutrophil priming remains elusive at this point. However, increased surface expression of FPR1 may lead to increased neutrophil responsiveness to its ligand, fMLF, the most important and well-studied neutrophil activator. Stimulation of neutrophils with fMLF leads, amongst other responses, to the release of chemokines, with CXCL8 (IL-8) being crucial for this response. CXCL8 functions as a chemoattractant to other cells, thereby directing them to the site of infection. Indeed, pre-incubation of human neutrophils with [6]-gingerol and subsequent stimulation with fMLF led to an increased secretion of the chemokine CXCL8. However, this effect could only be observed in a concentration range of 0.3–1 nM fMLF. The reason for this could be that higher fMLF concentrations induce receptor desensitization or internalization, which would abolish the [6]-gingerol-induced increase in FPR1 expression.[29] The increased response towards fMLF was also shown for ROS production after pre-incubation of the cells with [6]-gingerol and subsequent stimulation with 1 nM fMLF. Pharmacological inhibition of TRPV1 by trans-tert-butylcyclohexanol led to even lower ROS levels than in the control, which might indicate a general partial involvement of TRPV1 in ROS production in human neutrophils, like it was shown for mouse dorsal root ganglion neurons.[30]

The 2 h pre-incubation itself did not increase ROS production, which also points to an enhanced responsiveness of the cells, but no initiation of ROS production by [6]-gingerol. In addition, the cytokine and chemokine screening revealed only a quite low number of transcripts regulated by incubating neutrophils with [6]-gingerol, with even unchanged TRPV1 transcript and surface protein levels. This implies, that the observed functional and phenotypic changes occur in the absence of de novo synthesis.

With regard to somewhat inconsistent previous findings, our results support a functional expression of TRPV1 in human neutrophils. However, Schepetkin et al.[22] showed a TRPV1-mediated inhibitory impact of geranylacetone on neutrophil

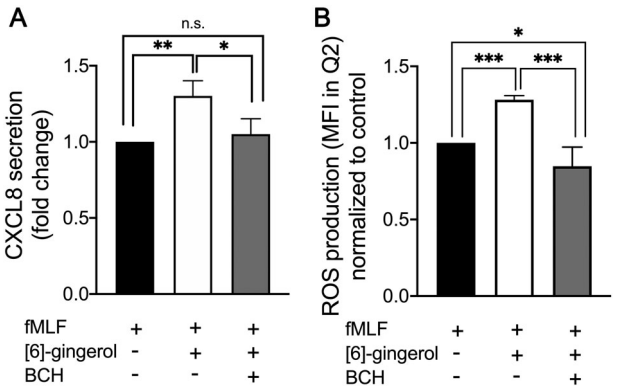

Figure 8. Pharmacological inhibition of TRPV1 reverses [6]-gingerol induced CXCL8 release and ROS production upon stimulation with fMLF. Isolated human neutrophils were incubated for 2 h with either 50 nM [6]-gingerol, 50 nM [6]-gingerol + 100 µM *trans-tert*-butylcyclohexanol (BCH), or 0.005% DMSO and 0.005% DMSO + 0.1% Ethanol as solvent controls, respectively. The solutions were washed out and the cells were further incubated with 1 nM fMLF for 4 h. Afterwards, CXCL8 concentrations in the supernatant were analyzed via ELISA A), or the cells were stained for ROS and analyzed via flow cytometry B). Data are shown as mean ± SD of three individual experiments. Statistical differences were calculated by one-way ANOVA followed by a Tukey's multiple comparisons test as post-hoc test. n.s.: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

migration towards fMLF, and a CXCL8-induced intracellular Ca^{2+} mobilization, in contrast to our results which point to a stimulatory effect on TRPV1 upon subsequent activation. Besides being different compounds, one reason for this discrepancy might be the different compound concentrations used, since Schepetkin et al.[22] applied a concentration of 50 µM geranlyacetone, whereas in our work we applied only 50 nM [6]-gingerol. This concentration was chosen based on our previous work, which showed a maximum plasma concentration of 42.0 ± 16.3 nmol L^{-1} at 30 min after ginger tea consumption and decreasing concentrations over a subsequent time course of 2 h. However, the ginger tea was consumed within 20 min, which may not correspond to usual consumption behavior. Therefore, we assumed that a plasma concentration of 50 nM [6]-gingerol is reachable via dietary intake, whereas higher concentrations are not very likely to occur.[9]

The inhibitory effect of geranylacetone was attributed to cross-desensitization, which would lead to decreased responses towards activating stimuli, and one might assume that this effect is only present at higher TRPV1 ligand concentrations in the µM range, leading to higher intracellular Ca^{2+} concentrations. In this context, it is worth mentioning that concentration response analyses in our previous work regarding cytokine secretion in T cells revealed an IC_{50} value of 82.2 µM for [6]-gingerol.[9] This, together with the observed inhibitory effect of 50 µM geranylacetone, would strengthen the assumption that high concentrations of TRPV1 ligands act rather inhibitively, whereas lower concentrations augment immune cell functions. [6]-gingerol concentrations below 50 nM were not included in the study. Therefore, the question remains, whether lower concentrations would reveal significant, biologically relevant effects. Nonetheless, the study presented demonstrates, that a concentration of [6]-gingerol, reached in the blood plasma after habitual dietary intake of ginger tea, is sufficient to facilitate CXCL8 secretion as well as ROS production in human neutrophils.

Our findings show that at nutritionally relevant concentrations, a sensory active food ingredient can affect general cellular responses in non-sensory tissue such as blood leukocytes via the TRPV1 channel. The observed modifications of neutrophil function, together with the extensive expression of TRP channels in blood leukocytes as potential target structures, underline the importance of assessing the bioactive potential of food ingredients with a particular focus on concentrations achievable through common dietary habits.

4. Experimental Section

Isolation of Leukocytes: Commercial blood samples from anonymized healthy donors were received from Sonnen-Gesundheitszentrum/Transfusionsmedizin, (English translation: Sun Health Center/Transfusin Medicine) Munich (Germany). Neutrophils, NK cells, B cells, and T cells were isolated using the respective MACSxpress Whole Blood Isolation Kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' protocol. Afterwards, remaining erythrocytes were depleted using the MACSxpress Erythrocyte Depletion Kit (Miltenyi Biotec). For the isolation of monocytes, the StraightFrom Whole Blood CD14 Micro Beads, the Whole Blood Column Kit, and Red Blood Cell Lysis Solution (Miltenyi Biotec) were used.

Isolation of RNA, Reverse Transcription, and Quantitative Real-Time PCR: RNA from leukocytes was isolated using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) following a DNase treatment using the RNase-free DNase set (Qiagen) according to the manufacturers' protocol. RNA integrity was determined via the RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) using the Bioanalyzer 2100 (Agilent Technologies). RNA was transcribed into cDNA using the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, Feldkirchen, Germany). For qPCR 50 ng of cDNA and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories GmbH) were mixed with specific primer pairs. The respective primers were validated via sequencing PCR products derived from qRT-PCR experiments using RNA from human brain or testis (from the FirstChoice Human Total RNA Survey Panel, Thermo Fisher Scientific) for TRPM4, TRPM5, and TRPV5, respectively. Primer and the respective product sequences were given in Table S1, Supporting Information. ACTB, B2M, HMBS, and HPRT1 were used as reference genes and the respective primers were purchased from Bio-Rad Laboratories. RT control, PCR control, gDNA control, and RNA quality control (Bio-Rad Laboratories) were used as general PCR controls. PCR reactions were performed on a CFX 96 Real-Time System (Bio-Rad Laboratories) using the following PCR conditions: 95 °C for 1 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Frequencies, which indicated in how many of the donors investigated the transcript could be detected, and Δct values were calculated using Microsoft Excel.

Incubation of Neutrophils: After isolation from blood of healthy donors, neutrophils were suspended in RPMI 1640 (Gibco, Thermo Fisher Scientific, Schwerte, Germany) containing no further supplements with a cell density of 1×10^6 mL^{-1}. [6]-gingerol (Sigma, ≥98% purity) or DMSO, respectively was added in a final concentration of 50 nM or 0.02%, respectively. Afterwards, the cells were incubated at 37 °C and 5% CO_2 for 2 h, and either used directly for further analyses or centrifuged at 300 × *g* for 10 min at room temperature, washed with RPMI 1640 (Gibco) and further incubated with fMLF (Alomone Labs, Jerusalem, Israel) for 4 h or DMSO (0.02%) as solvent control, respectively.

Immunostaining: For immunostaining, 10^5 neutrophils, either treated or non-treated, were centrifuged at 300 × *g* for 10 min. The supernatant was aspirated and the cells resuspended in PBS buffer. The antibodies were added according to the manufacturer, the suspensions mixed, and incubated at 4 °C for 10 min in the dark. Afterwards, the cells were washed using 1 mL of PBS buffer and resuspended in 500 µL of PBS buffer for

analysis by means of a MACSQuant Analyzer 16 (Miltenyi Biotec). Antibodies used were: CD15-VioBlue (final concentration: 2.5 µg mL^{-1}), CD62L-VioGreen (final concentration: 7.5 µg mL^{-1}), CD11b-APC (final concentration: 2 µg mL^{-1}), CD66b-APC/Vio770 (final concentration: 1 µg mL^{-1}), fMLP receptor antibody-FITC (final concentration: 20 µg mL^{-1}) (Miltenyi Biotec), anti-TRPV1-FITC (final concentration: 25 µg mL^{-1}) (Alomone Labs), Rabbit IgG Isotype Control-FITC (final concentration: 25 µg mL^{-1}) (Thermo Fisher Scientific). Cell viability was assessed using propidium iodide staining (Miltenyi Biotec). Mean fluorescence intensity (MFI) values and the corresponding figures were obtained by using the Flowlogic 7.3 software (inivai Technologies, Mentone, Victoria, Australia).

ROS Measurement: Reactive oxygen species were detected using the CellROX Green Reagent (Thermo Fisher Scientific) according to the manufacturers' instructions. Briefly, neutrophils were treated with either 50 nM [6]-gingerol for 2 h or 50 nM [6]-gingerol for 2 h and subsequently with 1 nM fMLF for 4 h, or the respective solvent controls. The CellROX reagent was added to the cells at a final concentration of 5 µM 30 min before the end of the incubation period and the mixture was further incubated for 30 min at 37 °C and 5% CO_2. Afterwards, the medium was removed, the cells were washed three times with PBS and analyzed by means of a MACSQuant Analyzer 16 (Miltenyi Biotec). Mean fluorescence intensity (MFI) values were obtained by using the Flowlogic 7.3 software (inivai Technologies).

Spectrofluorimetry: For measuring intracellular Ca^{2+} concentrations, neutrophils were suspended in RPMI 1640 (Gibco) with a cell density of 1×10^6 mL^{-1}. The cells were loaded with Fura-2 AM (Promocell, Heidelberg, Germany) for 45 min at 37 °C and 5% CO_2, washed and resuspended in RPMI 1640 (Gibco). The fluorescence of the cells was assessed using a SAFAS Xenius XC Spectrofluorometer (SAFAS, Monaco) at the following wavelengths: 340 nm excitation/510 nm emission and 380 nm excitation/510 nm emission. Fura-2 M is a ratiometric dye that can be excited at 340 nm for its calcium bound form and at 380 nm for its unbound form. An increase in the intracellular calcium concentration would lead to an increased fluorescence for the calcium bound form and a decreased fluorescence for the unbound form. The 340/380 ratio, which was used to account for unequal cellular loading of the dye, was calculated using Microsoft Excel. The 340/380 ratio for the cells treated with the ionophore ionomycin after 2 h was set to 100%, and the ratio for the cells treated with 50 nM [6]-gingerol and the solvent control was referred to this.

Enzyme-Linked Immunosorbent Assay: The CXCL8 concentrations in the supernatants derived from the incubation experiments (Section 4.3) were determined via Sandwich ELISA (DuoSet, R&D Systems, bio-techne, Minneapolis, MN, USA) according to the manufacturers' protocol.

Statistical Analyses: All experiments were performed using cells from at least three independent donors. Unless otherwise stated, data were shown as mean ± standard deviation (SD) calculated by GraphPad Prism 9.0. For the analyses of statistically significant differences, two sample Student's *t*-tests, or, in case more than two variables were compared, a one-way ANOVA followed by a Tukey's multiple comparisons test as post-hoc test was performed. The respective test used was indicated in the corresponding figure legend. A p value of ≤ 0.05 was considered statistically significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The Data Availability Statement was added on February 20, 2023.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

G.A., D.K., V.S. conceived and planned the experiments; G.A. and K.K. carried out the experiments; V.S. supervised the project; G.A. wrote the first draft of the manuscript. All authors reviewed the final manuscript.

Data Availability Statement

Data available on request from the authors.

Keywords

food, ginger, immune system, immunomodulation, polymorphonuclear leukocytes (PMNs)

Received: July 1, 2022
Revised: December 13, 2022
Published online: January 26, 2023

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