TRPM2 ion channels steer neutrophils towards a source of hydrogen peroxide

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Neutrophils must navigate accurately towards pathogens in order to destroy invaders and thus defend our bodies against infection. Here we show that hydrogen peroxide, a potent neutrophil chemoattractant, guides chemotaxis by activating calcium-permeable TRPM2 ion channels and generating an intracellular leading-edge calcium “pulse”. The thermal sensitivity of TRPM2 activation means that chemotaxis towards hydrogen peroxide is strongly promoted by small temperature elevations, suggesting that an important function of fever may be to enhance neutrophil chemotaxis by facilitating calcium influx through TRPM2. Chemotaxis towards conventional chemoattractants such as LPS, CXCL2 and C5a does not depend on TRPM2 but is driven in a similar way by leading-edge calcium pulses. Other proposed initiators of neutrophil movement, such as PI3K, Rac and lyn, influence chemotaxis by modulating the amplitude of calcium pulses. We propose that intracellular leading-edge calcium pulses are universal drivers of the motile machinery involved in neutrophil chemotaxis.

The first line of defence against pathogen invasion is formed by tissue-resident “sentinel” macrophages that detect characteristic pathogen-associated molecular patterns, engulf the invading pathogens and then kill them, partly by generating a “respiratory burst” of toxic free radicals. Following interaction with pathogens, sentinel macrophages release a range of chemoattractant signals, including cytokines and chemokines such as IL1, TNFα and CXCL2. In addition, destruction of bacterial proteins releases chemoattractant signals such as fMLP, and activation of the complement cascade releases signals such as C5a. In response to the release of these chemoattractants, circulating neutrophils leave the blood space and navigate up the chemoattractant gradients to locate and attack invading pathogens. A subsequent wave of macrophages and lymphocytes, such as natural killer (NK) T cells, joins the attack in a similar way1.

While these key features of the early response of the innate immune system have been known for some time, important questions remain. Are other chemoattractants also important? Hydrogen peroxide (H₂O₂) is a neutrophil chemoattractant in vitro2, and more recent in vivo evidence shows that H₂O₂, generated by the reaction with water of reactive oxygen species (ROS) produced by the NADPH oxygenase DUOX, forms a concentration gradient extending more than 100 μm from a site of injury, and moreover that H₂O₂ is a critical early guidance signal for neutrophil chemotaxis in vivo3–11. How does H₂O₂ act as a neutrophil chemoattractant? More generally, what are the cellular signalling mechanisms that guide neutrophils up gradients of chemoattractant molecules? The present study aimed to elucidate the mechanisms that couple gradients of H₂O₂ and other chemoattractants to neutrophil chemotaxis.

**Results**

**Hydrogen peroxide is a potent neutrophil chemoattractant both in vivo and in vitro.** We used a range of methods, both in vivo and in vitro, to confirm that H₂O₂ is a neutrophil chemoattractant. H₂O₂ injected into the peritoneal space in vivo recruits mouse neutrophils, with efficacy observed down to a concentration of 10 nM (Fig. 1A). Recruitment remains significantly elevated at concentrations between 10 nM and 10 μM but is inhibited at 100 μM. The well-established neutrophil chemoattractant fMLP also potently recruits neutrophils, with a similar efficacy to optimal concentrations of H₂O₂.
Figure 1. Neutrophil chemotaxis by H$_2$O$_2$ depends on TRPM2. (A) Chemotaxis of neutrophils into the peritoneum of WT and TRPM2$^-$/- mice following i.p. injection of H$_2$O$_2$. Direct injection of 0–100 μM H$_2$O$_2$ or 1 μM fMLP into the peritoneum of WT and TRPM2$^-$/- mice (see “Methods” section for details) elicited movement of total number of cells per peritoneum (ordinate). Time course of chemotaxis was determined in WT mice as shown in Supplementary Materials Fig. 1. Maximal chemotaxis in WT mice was calculated from the average of 3 points between 60 and 120 min. For TRPM2$^-$/- mice, chemotaxis was measured only at the 90 min point. Each bar shows mean ± SEM (n = 3 mice). (B) Boyden chamber assay of mouse neutrophil chemotaxis. Each bar shows mean ± SEM % migration from n = 4 mice (n = 8 for 0 µM H$_2$O$_2$). (C) In vitro ibidi μ-slide chemotaxis chamber assay using mouse neutrophils. Average speed of neutrophil migration, calculated for each neutrophil as total path length of distance travelled divided by time. Each bar shows mean ± SEM of n = 4 mice. (D) In vitro ibidi μ-slide chemotaxis chamber assay using mouse neutrophils. Forward migration index (FMI) is distance migrated in direction of gradient of H$_2$O$_2$ or fMLP divided by total distance travelled, from same experiments as shown in (C). Note that concentrations of H$_2$O$_2$ in the nanomolar range are able to increase both speed and directionality of neutrophil migration, and that migration is inhibited at high concentrations of H$_2$O$_2$ (> 10 μM). Distribution of values of FMI shown in Supplementary Materials Fig. 2. Each bar shows mean ± SEM of n = 4 mice. (F) In vitro ibidi μ-slide chemotaxis chamber assay using neutrophils isolated from human volunteer blood. FMI of human blood neutrophils shows similar dependence on concentration of H$_2$O$_2$ to mouse peritoneal neutrophils (compare with D). Each bar shows mean ± SEM of n = 3 individual human neutrophil samples. (F) Extracellular ADPR is a potent chemoattractant dependent on TRPM2 activation. WT mouse neutrophils showed significantly increased FMI compared to TRPM2$^-$/- neutrophils in response to 1–100 μM ADPR. Each bar shows mean ± SEM from n = 3 mice. Statistical analysis: For comparison with 0 H$_2$O$_2$: *, p < 0.05, **, p < 0.01, ###, p < 0.001, ####, p < 0.0001 (One-way ANOVA and Tukey–Kramer post-hoc test). For comparison with TRPM2$^-$/- (except in E): *, p < 0.05, **, p < 0.01, ***, p < 0.001, ****, p < 0.0001 (Two-way ANOVA and Bonferroni’s post-hoc test).
We next used the classical Boyden chamber method to measure neutrophil recruitment by H$_2$O$_2$ in vitro. In this assay, a membrane with 3 µm pores, through which only neutrophils can penetrate, separates the neutrophil and chemotactic compartments. H$_2$O$_2$ down to 1 nM concentration caused significant neutrophil recruitment through the membrane, with maximal recruitment between 10 and 10 μM H$_2$O$_2$ and a similar inhibition to that observed in vivo at 100 μM (Fig. 1B).

Similar results were obtained using a second in vitro method, the ibidi µ-slide, in which neutrophil motion is continuously tracked under a microscope (Fig. 1C,D and Supplementary Video 1). Figure 1C shows the speed of movement of neutrophils as a function of concentration of H$_2$O$_2$. Neutrophils undergo random movements in the absence of H$_2$O$_2$, so the average speed is not zero in the absence of H$_2$O$_2$ even though there is no significant directed movement (see Fig. 1D). The average speed is increased in a gradient of 1 nM H$_2$O$_2$ distributed over 1 mm, the distance between the compartments containing H$_2$O$_2$ and DMEM. Average speed is maximal between 0.1 and 10 μM H$_2$O$_2$ and is inhibited at 100 μM H$_2$O$_2$. Maximal speed in fMLP is not significantly different from that in H$_2$O$_2$.

An alternative method of quantifying neutrophil movement is to measure the forward migration index (FMI), a measure of directionality, that gives the ratio of linear distance travelled in the direction of the H$_2$O$_2$ gradient to total distance travelled (Fig. 1D). A significant increase in FMI was observed at 10 nM H$_2$O$_2$, and FMI was maximal between 0.1 and 10 μM H$_2$O$_2$. As in the other assays, directed migration was inhibited at 100 μM H$_2$O$_2$.

All three independent measures agree in showing that H$_2$O$_2$ is a highly potent neutrophil chemoattractant, from low nanomolar levels up to 10 μM, and that chemoattraction is inhibited at concentrations of H$_2$O$_2$ above 10 μM. A similar concentration-dependence of directed migration in a gradient of H$_2$O$_2$ was also observed with human blood neutrophils (Fig. 1E).

**Sensitivity of neutrophil chemotaxis to single molecules of H$_2$O$_2$.** The evidence above shows that neutrophils exhibit a clear increase in both movement and direction-finding in concentration gradients as low as 10 nM H$_2$O$_2$, distributed over the 1 mm gap between the chemotactic and DMEM compartments of the ibidi µ-slide. This gradient corresponds to a concentration difference between the leading and trailing edge of a 10 μm diameter neutrophil of 100 pM, and, assuming that H$_2$O$_2$ equilibrates rapidly and completely between the extracellular and intracellular spaces, this is a difference on average of around five intracellular molecules of H$_2$O$_2$ between the leading and trailing halves of the neutrophil (see calculation in legend to Supplementary Materials Fig. 2). The most sensitive neutrophils are therefore able to detect and respond reliably to only a few molecules difference of intracellular H$_2$O$_2$ between the leading and trailing halves of the cell. This extraordinary sensitivity to H$_2$O$_2$ suggests that the detection of H$_2$O$_2$ is a highly-evolved property, critical for survival.

**Neutrophil chemotaxis towards H$_2$O$_2$ depends on TRPM2.** The TRPM2 ion channel is expressed in neutrophils, and is potentely activated by H$_2$O$_2$, suggesting that it may be a candidate for the sensor of H$_2$O$_2$. H$_2$O$_2$ is thought to act indirectly to generate ADPR, which in turn directly activates TRPM2. TRPM2$^{−/−}$ mice are highly susceptible to infection, suggesting that the absence of TRPM2 causes a critical immune system defect. We found that genetic deletion of TRPM2 strongly inhibited H$_2$O$_2$-dependent neutrophil migration in all three assays (Fig. 1A–D), supporting the idea that TRPM2 is the major physiological sensor of H$_2$O$_2$ in neutrophils.

**Neutrophil chemotaxis towards ADPR depends on TRPM2.** The intracellular C terminus of TRPM2 contains a nudix hydrolase homology domain (NUDT9-H), named after the mitochondrial ADP-ribose (ADPR) pyrophosphatase NUDT9, to which ADPR binds to activate the ion channel. We found that neutrophils responded vigorously to an extracellular gradient of ADPR (Fig. 1F). Migration up a gradient of ADPR was strongly inhibited by deletion of TRPM2 (Fig. 1F), showing that neutrophil migration up a gradient of ADPR also depends on TRPM2. These experiments suggest that externally applied ADPR may be able to cross the cell membrane and activate the intracellular TRPM2 binding site. The idea that a large and hydrophilic molecule such as ADPR may be able to cross the cell membrane may seem unlikely, but it is supported by the activation of a TRPM2-dependent calcium influx when ADPR is applied externally (see Fig. 3G below), and also by work in other labs. It is possible that a specific carrier could transport ADPR across the cell membrane in order to activate the intracellular TRPM2 binding site.

**Chemotaxis towards most conventional chemoattractants does not depend on TRPM2.** The marked inhibition of neutrophil migration up gradients of H$_2$O$_2$ or ADPR, caused by deletion of TRPM2, could be due to an effect of the deletion on migratory ability per se. This possibility is not, however, supported by the observation that TRPM2 deletion does not affect chemotaxis to the known chemoattractants lipopolysaccharide (LPS), C5a and CXCL2 (Fig. 2A). Deletion of TRPM2, however, reduces but does not completely abolish chemotaxis towards the bacterial/mitochondrial peptide fMLP (Fig. 2A), as also noted in all assays shown in Fig. 1A–D, suggesting that fMLP exerts its chemotactic effect in part by activating TRPM2.

**Neutrophil chemotaxis towards H$_2$O$_2$ or ADPR is inhibited by blocking TRPM2.** To test further the dependence of neutrophil migration on TRPM2, we examined the effect of pharmacological block of TRPM2 with the inhibitor ACA (N-(p-aminocinnamoyl)anthranilic acid). Block of TRPM2 with ACA caused a significant inhibition of chemotaxis in response to both H$_2$O$_2$ and ADPR (Fig. 2B and Supplementary Materials Fig. 1B), similar in magnitude to the effect of genetic deletion of TRPM2 shown in Fig. 1. Block of TRPM2 with ACA had no effect, however, on chemotaxis towards LPS, C5a and CXCL2, but caused a partial inhibition of migration towards fMLP (Fig. 2B). ACA has off-target actions, but the close resemblance to results obtained...
with genetic deletion of TRPM2 supports the hypothesis that the effects of both genetic deletion and pharmacological block of TRPM2 are due to inhibition of TRPM2 itself and not to an off-target effect on another protein.

Figure 2C shows similar experiments on human blood neutrophils. All chemoattractants strongly increased FMI to a similar extent, as was also observed with mouse peritoneal neutrophils (Fig. 2A,B). In Fig. 2D, block of TRPM2 with ACA in human blood neutrophils largely inhibited migration up a gradient of H$_2$O$_2$ but had no effect on migration up a gradient of IL-8, the human homologue of CXCL2, results closely resembling those in mouse neutrophils (Fig. 2B).

In summary, the results in Figs. 1 and 2 strongly support the hypothesis that TRPM2 is the cellular target that mediates guidance of neutrophils up a gradient of H$_2$O$_2$.

Variability in values of FMI. There is a range of sensitivities to H$_2$O$_2$ amongst neutrophils, with some showing strongly directional migration up a gradient of H$_2$O$_2$ (FMI close to 1.0), while others show lower, or even absent, directional motility (Supplementary Materials Fig. 2 and Supplementary Video 1). The variability in values for FMI amongst individual neutrophils explains why the mean FMI does not typically exceed a value of around 0.6, when a value of 1.0 would be expected if all neutrophils migrated in a straight line. The proportion of highly mobile to less mobile neutrophils was found to be variable in different experiments (Supplementary Materials Fig. 2). The reason for this diversity in response is unknown but could be related to the age or condition of different neutrophils in the pool.
We tested whether the apparently lower maximal values of FMI in some experiments (for example with ADPR, Fig. 1F) was due to a real difference or simply reflected variability between different neutrophil samples. Figure 2A–C show that maximal FMI values are in fact not statistically different amongst H2O2, ADPR, LPS, C5a CXCL2 and fMLP when measured on a single neutrophil sample.

**Neutrophil migration to H2O2 depends strongly on temperature.** TRPM2 is a member of the large TRP family of ion channels, and like several other members of this family, is strongly activated by increasing temperatures within the physiological range12–15. Figure 3A shows that neutrophil migration towards very low concentrations of H2O2 is potently enhanced by small increases of temperature and that the effect of temperature is abolished by deletion of TRPM2. The effect of temperature is particularly prominent in a gradient of 1 nM H2O2, when an elevation from 37 to 39 °C, corresponding to a mild fever, enhances the ability of neutrophils to migrate up a gradient of H2O2 from an FMI of 0.052 ± 0.028 to 0.277 ± 0.059, a 5.3-fold increase. By contrast, the effect of temperature on migration towards the conventional chemoattractants CXCL2, C5a and LPS is much less marked (Fig. 3B–D). The strong temperature-dependence of neutrophil migration towards H2O2 provides further support for a critical role of TRPM2 in neutrophil migration in response to H2O2, and moreover suggests a reason why fever may be beneficial in fighting infection, because an elevation of temperature by only 2 °C, corresponding to a mild fever, gives a five-fold increase in neutrophil migratory ability towards low levels of H2O2.

**Temperature and chemoattractants elevate intracellular calcium by activating TRPM2.** TRPM2 is permeable to calcium ions14, like other members of the TRP family12,13, suggesting that the striking dependence of neutrophil migration on temperature may be due to a calcium influx through activated TRPM2 ion channels. Figure 3E shows that elevations of temperature in the physiological range (from 37 to 41 °C) induce an intracellular calcium increase in neutrophils, and that low concentrations of H2O2 (1 nM and 10 nM) enhance the amplitude of the calcium increase and lower its temperature threshold. Deletion of TRPM2 abolishes the thermally-induced elevations of calcium (Fig. 3F). The strong temperature-dependence of calcium influx through TRPM2 supports the hypothesis that a calcium influx through TRPM2 underlies the striking temperature-sensitivity of neutrophil migration driven by H2O2 (Fig. 3A). The results also support the idea that calcium influx through TRPM2 may be a driver of neutrophil motion, for which further evidence is presented below.

We next examined whether H2O2, ADPR and fMLP, whose effect on neutrophil migration depends on activation of TRPM2 (see above), also induce a calcium influx. Figure 3G and H show that in all cases these agonists cause an elevation of intracellular calcium, from the resting level of around 100 nM to 500 nM, and that the increase is dependent on TRPM2. There was a significant delay between application of H2O2 or ADPR and activation of calcium influx, consistent with their known intracellular sites of action16. H2O2 is able to cross the cell membrane through aquaporins10, and ADPR is also known to cross the neutrophil cell membrane, though the mechanisms are not clear25,26. There was a rapid drop in intracellular calcium when external calcium was removed (Fig. 3G, left-hand panel), showing that activation of TRPM2 causes an influx of calcium from the extracellular solution.

The above experiments using calcium imaging are in agreement with patch-clamp recordings both from HEK cells transfected with TRPM2 and from isolated TRPM2-expressing membrane patches, which show that TRPM2 is directly activated by heat and by intracellular ADPR13,15,16,30,31. Recordings from membrane patches have shown, however, that H2O2 does not directly activate TRPM216. The action of H2O2 on intact cells is therefore likely to be caused by generation of intracellular ADPR which then activates the TRPM2 ion channel (see diagram in Supplementary Materials Fig. 8).

**Leading-edge calcium pulses in H2O2 and ADPR are driven by TRPM2.** The results above suggest that preferential activation of TRPM2 channels, by elevated levels of H2O2 at the neutrophil leading edge, could cause a localised calcium influx that guides neutrophil chemotaxis by coupling to the motile machinery. We used calcium imaging of migrating neutrophils and we observed that an elevated intracellular calcium concentration at the neutrophil leading edge is indeed seen during movement of a neutrophil up a gradient of H2O2 (Fig. 4A, left panel). Leading-edge intracellular calcium elevations were seen in all migrating neutrophils, and they had a pulsatile appearance for which the name “calcium pulse” seemed appropriate (see for example Supplementary Video 2). The calcium pulse is most prominent at the base of the pseudopodium, but in some images it can be seen also invading the advancing pseudopodium itself (Figs. 4B, C and 5A, B). Genetic deletion of TRPM2 abolishes both calcium pulses and cell migration (Fig. 4A, centre, and Fig. 4B–E). At high concentrations of H2O2, maximal activation of TRPM2 over all of the cell membrane means that a uniformly high level of calcium floods the cell and neutrophil migration is abolished (Fig. 4A, right).

Pharmacological block of TRPM2 with the inhibitor ACA16 has a similar effect to genetic deletion of TRPM2 in inhibiting calcium pulses and neutrophil chemotaxis towards H2O2 and ADPR (Fig. 4B–E). Both calcium pulses and neutrophil movement are abolished by withdrawal of extracellular calcium (Fig. 4B, D), consistent with an influx of calcium from the external medium acting as a trigger for the generation of intracellular calcium pulses. Lowering the temperature to 33 °C, which strongly inhibits neutrophil motility (Fig. 3A), also inhibits the generation of calcium pulses (Fig. 4B, D and Supplementary Materials Fig. 4B). These parallel observations using a range of approaches support the idea that leading-edge calcium pulses in both H2O2 and ADPR are generated by an influx of calcium through TRPM2, and that calcium pulses are coupled to neutrophil migration.

**Leading-edge calcium pulses in classical chemoattractants are not driven by TRPM2.** Figure 5B and Supplementary Materials Fig. 5 show that leading-edge calcium pulses, similar to those observed in H2O2 and ADPR, are also seen in gradients of the chemoattractants CXCL2, LPS and C5a. These observations
suggestion that calcium pulses “steering” neutrophil chemotaxis, by determining the future direction of establishment of pseudopodia, may be a universal mechanism. Block of TRPM2 with ACA has no effect on calcium pulses in the classical chemoattractants CXCL2, LPS and C5a (Fig. 5B and Supplementary Materials Fig. 5). Together with the data in Fig. 2, showing the lack of effect of TRPM2 deletion or block on neutrophil migration to classical chemoattractants, these experiments show that neither the generation of leading-edge calcium pulses nor neutrophil migration in these chemoattractants is driven by a TRPM2-dependent mechanism.

**Signalling pathways determining neutrophil motility.** There have been several proposals regarding the mechanisms that couple chemoattractants to the intracellular motile machinery of immune cells, including activation of PI3K to generate PI(3)P at the neutrophil leading edge, leading to activation of Rac, or, in the case of chemoattraction by H₂O₂, oxidative modulation of cysteine residues present in the Src family tyrosine kinase lyn. Figure 5 and Supplementary Materials Figs. 5 and 6 investigate potential signalling pathways involved in neutrophil chemotaxis. PP2, a potent inhibitor of Src family kinases (SKF), caused only partial inhibition of motility in a gradient of H₂O₂ (Fig. 5A,C and Supplementary Materials Fig. 6) suggesting that a member of the Src kinase family modulates H₂O₂-directed motility but is not essential for generating movement. PP2 also partially inhibited the amplitude of calcium pulses (Fig. 5A,C), consistent with a SKF member acting upstream of both the generation of calcium pulses and cell movement. Interestingly, PP2 had no effect on either neutrophil motility or generation of calcium pulses in gradients of the classical chemoattractants CXCL2, LPS or C5a (Fig. 5B,D and Supplementary Materials Fig. 5, 6), showing that the effect of SKF inhibition is specific to the H₂O₂/TRPM2 mechanism. One possibility is that lyn may promote trafficking of TRPM2 to the surface membrane and thus that inhibition of lyn would reduce the calcium influx triggered when TRPM2 is activated by H₂O₂ by reducing surface membrane expression of TRPM2. In support of this proposal, an SKF member has been shown to play a similar role in promoting trafficking of TRPV1 to the neuronal cell surface membrane.

PI3K and Rac have also been suggested as initiators of neutrophil chemotaxis. However, the PI3K inhibitor wortmannin and the Rac inhibitor EHT1864 only partially inhibited neutrophil chemotaxis in response to H₂O₂, CXCL2, LPS and C5a (Fig. 5 and Supplementary Materials Figs. 5, 6). In the case of chemotaxis in a gradient of H₂O₂, the residual motility in the presence of the PI3K and Rac blockers was completely inhibited by the TRPM2 blocker ACA (Supplementary Materials Fig. 6A). The PI3K and Rac inhibitors also reduced the amplitudes of leading-edge calcium signals, suggesting that PI3K and Rac play a modulatory role in the pathways upstream of the generation of calcium pulses, but that the amplitude of calcium pulses is the primary event controlling motility in response to all chemoattractants.

**Calcium pulses determine the direction of neutrophil chemotaxis.** Increases in intracellular calcium in neutrophils and other immune cells have been suggested to be a consequence of cell movement, as might arise if a calcium increase was required to retract the trailing edge of the cell, or because an interaction between cell and substrate triggers calcium entry caused by the movement itself. In these scenarios, the calcium increase would be expected to follow the movement, while if the calcium pulse directs the movement it would be expected to precede it. For this reason we investigated whether a change in the cellular location of calcium pulses precedes a subsequent change in the direction of cell migration (calcium drives migration), or whether a change in the direction of migration precedes a change in location of calcium pulses (migration drives calcium). Neutrophils in the absence of a chemotactic gradient still exhibit calcium pulses, which change cellular location at intervals, and also exhibit random migration (See Supplementary Video 2), providing a useful way of distinguishing these two possibilities.
We find that neutrophils are guided by H$_2$O$_2$ over four orders of magnitude of concentration, from 1 nM to 10 µM. Higher concentrations of H$_2$O$_2$ (> 10 µM) inhibit chemotaxis and cause an elevated calcium concentration throughout the cell. In agreement, previous studies have also found inhibition of chemotaxis in neutrophils and lymphocytes at levels of H$_2$O$_2$ above 10 µM. A probable cause of the inhibition of chemotaxis at higher H$_2$O$_2$ is loss of the differential intracellular calcium gradient necessary for cell guidance, as shown in Fig. 4A.

Neutrophil guidance towards most conventional chemoattractants, such as LPS, C5a or CXCL2, is independent of TRPM2, but similar leading-edge calcium pulses are still observed, suggesting that guidance by calcium pulses is a universal mechanism. Signalling from G-protein coupled receptors activated by conventional chemoattractants to intracellular calcium stores is a likely mechanism (see diagram in Supplementary Materials).

**Discussion**

The work described here confirms previous studies showing that H$_2$O$_2$ is a potent neutrophil chemoattractant both in vitro and in vivo. We demonstrate an essential role for the TRPM2 ion channel in directing neutrophil chemotaxis towards H$_2$O$_2$. We propose that preferential activation of TRPM2 by the higher levels of H$_2$O$_2$ at the neutrophil leading edge causes a calcium influx, triggering amplified leading-edge calcium pulses that determine the direction of extension of pseudopodia and therefore of future neutrophil motion. There is a significant delay, with total half-time for the alignment of cell movement to a change in calcium direction of 146 s and 164 s. The neutrophil movement therefore reacts to a change in the direction of a calcium pulse with a surprisingly long delay, with total half-time of around 160 s, or almost 3 min.

Finally, Fig. 6D shows a comparison between the external H$_2$O$_2$ concentration surrounding a cell migrating half-way up a 10 nM gradient over 1 mm, and the internal calcium concentration measured with fura-2. The calcium gradient is very much steeper than the H$_2$O$_2$ gradient, showing that there must be a powerful non-linear amplification process that converts the gentle H$_2$O$_2$ gradient into the strongly enhanced calcium signal seen at the cell leading edge.
Figure 5. Effect of pharmacological blockers on neutrophil migration towards H$_2$O$_2$ and chemokine CXCL2. (A) Gradient of H$_2$O$_2$ (10 μM over 1 mm). From top: H$_2$O$_2$ alone; TRPM2 blocker ACA (N-(p-amylcinnamoyl) anthranilic acid, 10 μM) completely inhibits both migration and Ca pulses (reproduced from Fig. 4); migration is slowed and Ca$^{2+}$ pulses partially inhibited by the Src-family kinase inhibitor PP2 (10 μM), PI3kinase inhibitor wortmannin (100 nM) and Rac inhibitor EHT 1864 (50 μM). Images shown at 10 s intervals in each panel. Long blue arrows—movement of neutrophil a distance equal to or greater than the cell diameter; arrowheads—movement less than cell diameter; no arrow—no significant movement. Other details as in Fig. 4. Images representative of n = 3 individual experiments. (B) Similar experiment with gradient of CXCL2 (10 nM). Migration and Ca$^{2+}$ pulses are unaffected by ACA (10 μM) and PP2 (10 μM), while migration is slowed and Ca$^{2+}$ pulse amplitude reduced by wortmannin (100 nM) and ETH 1864 (50 μM). Images representative of n = 3 individual experiments. (C) Peak calcium and cell velocity for the conditions shown in (A). Bars show average of n = 3 individual cells. Two-way ANOVA and Tukey–Kramer post-hoc test compared to first bar. *, p < 0.05; ***, p < 0.001; ****, p < 0.0001, ns = not significant. Other details as in Fig. 4. (D) Similar plots for conditions shown in (B). Similar experiments for conventional chemoattractants C5a and LPS shown in Supplementary Materials Fig. 5.
Fig. 8). An interesting exception to the TRPM2-independence of conventional chemoattractants is chemotaxis towards the chemoattractant peptide fMLP, which is partially inhibited either by either pharmacological block or genetic deletion of TRPM2. The rapid elevation of intracellular calcium caused by fMLP (Fig. 3G) suggests that activation of FPR1, the receptor for fMLP, may directly activate TRPM2, in addition to coupling to downstream G protein-signalling pathways. This proposal is supported by the recently-discovered physical interaction between TRPM2 and the FPR1 receptor.45

Previous studies of the involvement of TRPM2 in neutrophil chemotaxis have come to contradictory conclusions. Yamamoto et al.49 found that deletion of TRPM2 decreased neutrophil migration in response to an inflammatory supernatant formed by activated neutrophils in vitro, and also decreased movement to areas of inflammation in vivo. In support, Hiroi et al.30 found that neutrophil invasion following myocardial ischaemia was lowered in Trpm2−/− mice. Wang et al.40, in contrast, found that deletion of TRPM2 promotes chemotaxis both in vitro and in vivo and they propose that TRPM2 generates a “stop” signal to halt neutrophil migration in the vicinity of pathogens. In the present study we show that neutrophil motility both in vitro and in vivo is potently promoted by low levels of H2O2, and that motility is inhibited, both in vivo and in vitro, by either pharmacological inhibition or genetic deletion of TRPM2. Thus, at low concentrations of H2O2, TRPM2 mediates a powerful chemoattractant mechanism. In addition, we find that high concentrations of H2O2 (above 10 µM) inhibit neutrophil motility, which may be critical in providing a “stop” mechanism in the vicinity of pathogens. We show that this inhibition is due to a rise of the internal calcium level throughout the cell when TRPM2 channels are maximally activated by H2O2, thus flooding the cell with calcium and abolishing the internal calcium gradient necessary for neutrophil navigation. Thus the apparently contradictory conclusions of previous studies can be reconciled by the data obtained in the present work: H2O2, acting via TRPM2, is a potent chemoattractant at low levels of H2O2, but at higher levels of H2O2 over-activation of TRPM2 floods the cell with calcium, abolishing the calcium gradient necessary to drive chemotaxis and halting cell movement.

The ratio between calcium at the leading and trailing edge of an advancing neutrophil is much greater than the ratio between H2O2 concentrations (Fig. 6D), suggesting that a process of non-linear amplification enhances the leading-edge calcium signal. One likely possibility for such a mechanism is the highly non-linear process of calcium-induced calcium release from subcellular stores.25,26,47,48,51

A scheme consistent with the work reported here is shown in Supplementary Materials Fig. 8. In a gradient of H2O2, preferential activation of TRPM2 ion channels at the leading edge causes an influx of calcium from the extracellular solution, triggering calcium-induced calcium release from intracellular stores. The Src family kinase lyn modulates this pathway at an early stage, perhaps by regulating trafficking of TRPM2 ion channels to the surface membrane. The intracellular signalling molecules PI3K and Rac, which have been proposed as drivers of chemotaxis, do not appear to play a direct role in chemotaxis, but instead fine-tune motility to all chemoattractants by regulating a step common to all, for example refilling of intracellular calcium stores, and thus modulate the amplitude of the leading-edge calcium pulses.

Some studies have found no evidence for calcium gradients in neutrophils during chemotaxis,44,52 though others have obtained evidence that leading-edge calcium signals play a critical role in chemotaxis of many different cell types.53,54 In the present work, we observed leading-edge calcium signals in all motile neutrophils in vitro. Interestingly, leading-edge calcium signals have also recently been detected in migrating neutrophils in vivo.55 These authors find that neutrophils can be guided in vivo by expressing TRPV1 and then imposing a directional gradient of the TRPV1 agonist capsaicin.55,56 This work supports the idea that a TRP channel alone can mediate chemotaxis both in vitro and in vivo and they propose that TRPM2 generates a “stop” signal to halt neutrophil migration in the vicinity of pathogens. We show that this inhibition is due to a rise of the internal calcium level throughout the cell when TRPM2 channels are maximally activated by H2O2, thus flooding the cell with calcium and abolishing the internal calcium gradient necessary for neutrophil navigation. Thus the apparently contradictory conclusions of previous studies can be reconciled by the data obtained in the present work: H2O2, acting via TRPM2, is a potent chemoattractant at low levels of H2O2, but at higher levels of H2O2 over-activation of TRPM2 floods the cell with calcium, abolishing the calcium gradient necessary to drive chemotaxis and halting cell movement.

Neutrophils are able to navigate up a gradient of H2O2 in which there is an average difference of only a few molecules of intracellular H2O2 between the leading and trailing halves of the cell. This extraordinary sensitivity suggests that navigation towards H2O2 is vital for mammalian survival and has been highly honed by evolution. The strong temperature dependence of TRPM2 activation28–30 suggests a novel function for fever: by enhancing the calcium gradient necessary for neutrophil navigation. Thus the apparently contradictory conclusions of previous studies can be reconciled by the data obtained in the present work: H2O2, acting via TRPM2, is a potent chemoattractant at low levels of H2O2, but at higher levels of H2O2 over-activation of TRPM2 floods the cell with calcium, abolishing the calcium gradient necessary to drive chemotaxis and halting cell movement.

The ratio between calcium at the leading and trailing edge of an advancing neutrophil is much greater than the ratio between H2O2 concentrations (Fig. 6D), suggesting that a process of non-linear amplification enhances the leading-edge calcium signal. One likely possibility for such a mechanism is the highly non-linear process of calcium-induced calcium release from subcellular stores.

A scheme consistent with the work reported here is shown in Supplementary Materials Fig. 8. In a gradient of H2O2, preferential activation of TRPM2 ion channels at the leading edge causes an influx of calcium from the extracellular solution, triggering calcium-induced calcium release from intracellular stores. The Src family kinase lyn modulates this pathway at an early stage, perhaps by regulating trafficking of TRPM2 ion channels to the surface membrane. The intracellular signalling molecules PI3K and Rac, which have been proposed as drivers of chemotaxis, do not appear to play a direct role in chemotaxis, but instead fine-tune motility to all chemoattractants by regulating a step common to all, for example refilling of intracellular calcium stores, and thus modulate the amplitude of the leading-edge calcium pulses.

Some studies have found no evidence for calcium gradients in neutrophils during chemotaxis,44,52 though others have obtained evidence that leading-edge calcium signals play a critical role in chemotaxis of many different cell types.53,54 In the present work, we observed leading-edge calcium signals in all motile neutrophils in vitro. Interestingly, leading-edge calcium signals have also recently been detected in migrating neutrophils in vivo.55 These authors find that neutrophils can be guided in vivo by expressing TRPV1 and then imposing a directional gradient of the TRPV1 agonist capsaicin.55,56 This work supports the idea that a TRP channel alone can mediate chemotaxis both in vitro and in vivo and they propose that TRPM2 generates a “stop” signal to halt neutrophil migration in the vicinity of pathogens. We show that this inhibition is due to a rise of the internal calcium level throughout the cell when TRPM2 channels are maximally activated by H2O2, thus flooding the cell with calcium and abolishing the internal calcium gradient necessary for neutrophil navigation. Thus the apparently contradictory conclusions of previous studies can be reconciled by the data obtained in the present work: H2O2, acting via TRPM2, is a potent chemoattractant at low levels of H2O2, but at higher levels of H2O2 over-activation of TRPM2 floods the cell with calcium, abolishing the calcium gradient necessary to drive chemotaxis and halting cell movement.

Materials and methods

Animals. Black C57BL/6 WT and TRPM2+/− K/O mice (6–8 weeks old) were bred in house from matings of TRPM2+/− heterozygous mice to ensure that the genetic and environmental backgrounds of both WT and TRPM2−/− mice were as far as possible identical. TRPM2+/− K/O mice were a gift from the Mori laboratory (Kyoto, Japan)68 and had been backcrossed onto the WT C57BL/6 strain for nine generations. For experiments in which comparisons with K/O were not required, Black C57BL/6 WT mice (6–8 weeks old) were purchased from Charles River Inc. All animal work was conducted under UK Home Office personal and project licences, approved by the Animal Welfare Ethical Review Board (AWERB) of King’s College London and carried out in accordance with The Animals (Scientific Procedures) Act 1986 and in compliance with the ARRIVE guidelines.

Chemicals and reagents. Hydrogen peroxide (H2O2, 31642), adenosine 5’diphosphoribose (ADPR, A0752), N-formylmethionyl-leucyl-phenylalanine (fMLP, F3506), thioglycolate (70157), lipopolysaccharide (LPS, LPS25), complement component 5α (C5a, SRP4985A), N-(p-Amylcinnamoyl)anthranilic acid (ACA, A8486), PP2 (P0042), interleukin-8 (IL-8, CXCL8 SRP3098) were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., Dorset, UK). Wortmannin (W1628) was purchased from Yorlab (York, NY).
specific 3 µm pores. Non-migrated cells were removed from the inserts and the inserts placed in cell-detachment solution for 60 s to dislodge cells. The peritoneal fluid was extracted and centrifuged for 10 min at 200 RCF. The supernatant was discarded and cells suspended in PBS injected into the peritoneal cavity and massaged gently for 60 s to dislodge cells. The peritoneal fluid (10 μl/g) and, after 4 h, euthanised by cervical dislocation. The peritoneal-covering skin was removed, 5 ml PBS injected into the peritoneal cavity and massaged gently for 60 s to dislodge cells. The peritoneal fluid was extracted and centrifuged for 10 min at 200 RCF. The supernatant was discarded and the cells suspended in DMEM (Thermo Fisher Scientific Cat. No. 41966-029) + 10% FBS. The total number of cells recovered was counted, using a Neubauer chamber, as a semi-quantitative measure of cell chemotaxis. Cell numbers were corrected for the amount of fluid recovered as a fraction of 5 ml.

**In vivo model: measurement of H₂O₂-induced peritoneal cell chemotaxis.** An in vivo model was developed in which WT and TRPM2 K/O mice were subjected to intraperitoneal (IP) injection (10 μl/g) of: 0, 0.001, 0.01, 0.1, 1, 10 or 100 μM H₂O₂ or 1 μM fMLP in H₂O. After a variable time mice were euthanised, skin covering the peritoneum was removed, 5 ml PBS injected into the peritoneal cavity and the cavity massaged gently for 60 s to dislodge cells. The peritoneal fluid was extracted and centrifuged for 10 min at 200 RCF. The supernatant was discarded and the cells suspended in DMEM + 10% FBS. This method generated cell suspensions containing > 90% of neutrophils, identified through cell identification in peritoneal extract. Cell suspension was isolated from peritonea of WT mice as above, spun down onto glass slides using a Cytospin at 400 RPM for 5 min and left to air-dry overnight. A modified version of the May-Grünwald-Giemsa staining (RAL555) was purchased from RAL Diagnostics, (RAL Diagnostics, Martillac, France).

**Isolation of mouse peritoneal neutrophils.** Mice were injected IP with 3% thioglycolate solution (10 μl/g) and, after 4 h, euthanised by cervical dislocation. The peritoneal-covering skin was removed, 5 ml PBS injected into the peritoneal cavity and massaged gently for 60 s to dislodge cells. The peritoneal fluid was extracted and centrifuged for 10 min at 200 RCF. The supernatant was discarded and cells suspended in DMEM + 10% FBS. This method generated cell suspensions containing > 90% of neutrophils, identified through cell identification in peritoneal extract. Cell suspension was isolated from peritonea of WT mice as above, spun down onto glass slides using a Cytospin at 400 RPM for 5 min and left to air-dry overnight. A modified version of the May-Grünwald-Giemsa staining was used to identify cell types (RAL DIFF-QUIK kit, RAL diagnostics). Slides were suspended in RAL Diff-Quick fixative solution (methanol based solution to stabilize cellular components) for 1 min, in RAL Diff-Quik solution I (Xanthene solution; a buffered solution of Eosin Y) for 1 min and in RAL Diff-Quik solution II (a buffered solution of thiazine dyes, consisting of methylene blue and Azure A) for 1 min. Nuclei were meta-chromatically stained red/purple and cytoplasm pink/yellow (See Supplementary Materials Fig. 9).

**Isolation of human blood neutrophils.** Blood samples were collected from healthy volunteers by Dr Lucy Norling at Queen Mary College, University of London, with written consent. Human cells were prepared according to a protocol approved by the East London & The City Local Research Ethics Committee (no. 06/Q605/40; P/00/029 ELCHA, London, United Kingdom) and kindly donated for use in our experiments. Blood was collected into 3.2% sodium citrate and diluted 1:1 in RPMI 1640 before separation through a double-density gradient as described.

**In vitro Boydne chamber neutrophil migration assay.** QCM Boyden chamber chemotaxis neutrophil migration assay kits were purchased from Merck Millipore (ECM505, Massachusetts, USA). Neutrophil chemotaxis cell migration assays were conducted according to manufacturer’s instructions. In brief, neutrophils, isolated and prepared as above were seeded at 2 × 10⁵ cells per insert and each insert placed in a chamber containing either DMEM medium alone, 0.001 μM, 0.01 μM, 0.1 μM, 1 μM, 10 μM, 100 μM H₂O₂ or 1 μM fMLP. Inserts and chambers were incubated at 37 °C in 95% air/5% CO₂ for 4 h to allow cells to migrate through neutrophil-specific 3 μm pores. Non-migrated cells were removed from the inserts and the inserts placed in cell-detachment solution for 60 s to dislodge cells.

**Figure 6.** Calcium pulses direct neutrophil chemotaxis. (A) Method for determining the relation between direction of calcium pulses and direction of neutrophil movement. Red vector (“calcium vector”) shows direction in which the calcium pulse is ‘pointing’, by connecting cell geometric centre to simultaneous location of maximal calcium signal, while grey or black vectors show direction of movement of cell geometric centre (“movement vector”), which can be calculated at earlier (1) or later times (2, 3). (B) Tracks of geometric cell centre (orange) and position of peak calcium concentration (blue) at 5 s intervals for a neutrophil moving in absence of chemotactic gradient. (See movie in Supplementary Video 2). Similar image series taken at a 10 × shorter intervals did not show any obvious difference in the dynamics of movement of peak calcium concentration (Supplementary Materials Fig. 8). Red vectors (shown at 100 s intervals) give direction of calcium vector (see A). Black vectors show movement vector (see A) connecting the geometric centre at the same time as each calcium vector with the geometric centre 50 s later. At the time indicated by the star the calcium vector shifts abruptly from an average upward orientation to an average downward orientation. There is a clear delay before the movement vector follows. (C) Calcium vector (blue) and movement vector (orange) angles for cell shown in (B). A delay (Δ) of 75 s best predicts the moment at which the movement vector begins to follow the abrupt change in direction of calcium vector. There is a further delay before the cell movement aligns with the new calcium vector orientation (half-time < 83 s), giving total delay between change of calcium vector and half-alignment of movement vector of 158 s. (D) Calcium concentration (blue), determined from fura-2 fluorescence ratio, shown in the in the direction of migration from left to right of a neutrophil located half-way up a gradient of H₂O₂ from 0 to 10 μM. For comparison, red line shows H₂O₂ concentration (in molecules of H₂O₂ per μm²) along the length of the cell. Horizontal axis gives distance from left-hand to right-hand edges of the cell, in the direction both of the H₂O₂ gradient and of cell motion.
solution for 30 min at 37 °C in 95% air/5% CO₂. 112 µl of this solution and of the lower chamber were added to
the same wells of black-walled 96 well plates. Cells were lysed in the wells and incubated with CyQUANT GR
dye, which emits increasing fluorescence proportional to the levels of bound nucleic acid released from the lysed
cells. Following a 15 min incubation at room temperature, fluorescence intensity was analysed on a FLUOstar
Omega microplate reader (BMG LABTECH, Buckinghamshire, UK) at 480/520 nm.

**Neutrophil chemotaxis assays.** Ibidi µ-slide chemotaxis assay chambers, precoated with collagen IV
along the central migration strip, were purchased from Thistle Scientific Ltd (Uddingston, Glasgow, UK). Neu-
traphils, isolated as above from peritonea of WT or TRPM2 K/O mice or from human blood, were re-suspended
within 30 min of collection in DMEM + 10% FBS at a concentration of 5 × 10^5 cells per ml and 6 µl was seeded
along the central migration strip of an Ibidi µ-slide chamber as per the manufacturer’s instructions. Slides were
incubated for 1 h at 37 °C in humidified 95% air/5% CO₂, to allow neutrophil adherence to the central migra-
tion strip. DMEM (without added FBS) with and without added chemotactrant was then added to the wells
on opposite sides of the central migration strip. DMEM was from Thermo Fisher Scientific Cat. No. 41966-029,
or for experiments in which the effects of calcium removal were studied, 0Ca DMEM was from Thermo Fisher
Scientific Cat. No. 21068–028. For experiments in which effects of inhibitors were to be tested, equal concentra-
tions were added to both DMEM + chemotactrant and DMEM wells. Slides were pre-incubated at 37 °C in 95%
air/5% CO₂ for 20 min to allow the generation of a gradient of chemoattractant across the 1 mm wide × 70 µm
depth central cell migration strip. Live-cell time-lapse microscopy was then conducted using a 10 × lens and dark-
field illumination on a Nikon Eclipse Ti-E inverted microscope equipped with the Nikon Perfect Focus System
(PFS). The images shown are centered on the middle of the 1 mm wide cell migration strip and span the majority
of its width. The microscope was housed in a temperature-controlled Perspec box (Solenit Scientific) at 37 °C,
or other temperatures as desired, with slides housed in a stage-mounted block in humidified 95% air/5% CO₂.
A maximum of 12 individual chambers (4 individual slides, 3 chambers per slide) could be imaged per exper-
iment by using a motorized stage. Stage movement, lens focus and image acquisition were controlled by Nikon
NIS Elements software. Experiments were conducted over 2 h, with images of each assay compartment taken
every 2 min. The ImageJ Fiji TrackMate plug-in was employed to track individual neutrophils. A chemotaxis and
migration plug-in, provided by ibidi, was used to calculate speed and forward migration index (FMI) data from
the neutrophil tracks.

**Calcium imaging of neutrophils.** Neutrophils isolated as above were re-suspended in DMEM + 10% FBS
at a concentration of 5 × 10^5 per ml.

For experiments in which intracellular calcium was to be recorded while extracellular solutions were changed,
neutrophils were plated onto a collagen-coated 13 mm round glass coverslip and incubated at 37 °C in 95%air/5%
CO₂ for 1 h to allow neutrophils to adhere. Fura2-AM (5 uM in DMEM) was then added to the cells on the cover-
slip for 30 min at 37 °C in 95% air/5% CO₂. Fluorescence was measured during alternating illumination at 340 nm
and 380 nm (OptoScan; Cairn Research Inc, Kent, UK) every 2 s using a Nikon Eclipse Ti inverted microscope
with a 40 × lens and iXon 897 EM-CCD camera controlled by WinFluor 3.2 software. F_340/380 ratios were obtained
using FIIJ (ImageJ) and converted to calcium concentrations using the equation given by Grynkiewicz et al. with values R_{max} = 2.501, R_{min} = 0.103, both determined experimentally, see Fig. 3G. Full details in Vilar et al.31

For experiments when calcium signals during chemotaxis up a gradient of chemoattractant were to be
recorded, 1 µl of Fura-2 AM solution (50 µg Fura-2 AM + 10 µl pluronatic F-127 + 10 µl DMEM) was added to
500 µl of neutrophil suspension and incubated for 1 h at 37 °C in 95%air/5% CO₂. Fura-2 loaded cells in suspen-
sion were seeded into ibidi chambers as previously and imaged in a Nikon Ti-E microscope with a 40 × phase
contrast lens. Fast-moving neutrophils located in the middle of the central cell migration strip were selected, with
typically only one cell imaged per field. Calcium ratio images were obtained with alternating 340 nm and 380 nm
epi-illumination supplied by stable LED light sources (Fura-LED, Cairn Research), typically at 5 s intervals. In
some experiments acquisition of each pair of epifluorescence images was followed by a transmitted white-light
phase-contrast image. All images were filtered by a broad-band 510 nm filter and captured with a Photomet-
rics Prime 95B sCMOS camera. Stage movement, focus and image acquisition were controlled by Nikon NIS
Elements software. The ImageJ Fiji RatioPlus plug-in was used to generate F_340/380 ratio images and a rainbow
look-up table (LUT) was applied to the ratio images to indicate the level of calcium. Values of the ratio F_340/380
were converted to intracellular free calcium concentration using the equation given by Grynkiewicz et al.59. To
determine the values of fluorescence ratios at maximal (R_{max}) and zero (R_{min}) calcium levels, neutrophils were
imaged in the presence of ionomycin (10 µM) either in DMEM or in 0Ca DMEM with added 2 mM EGTA, giv-
ning mean values R_{max} = 3.132 and R_{min} = 0.803 (note that the differences between these values and those above
arises because chemotaxis experiments were performed on different apparatus). To indicate the location of the
maximum calcium level on a phase-contrast image, a 3-colour LUT was applied to F_340/380 images using the Fiji maximum intensity
identifying tool. The coordinates for the geometric centre of the neutrophil were identified using the cell edge and
centre localising tool. In some images (for example, Fig. 4A) mean values of F_340/380 in leading and trailing
halves was calculated by identifying the cell outline using the cell edge localising tool and dividing the cell by
hand into leading and trailing halves in the direction of the imposed chemotactic gradient.

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Author contributions

H.M. and S.L. performed experiments shown in the MS. C.-H. T. and V.S. performed preliminary experiments. H.M. prepared figures. PMcN directed the project and wrote the text. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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