occluded the effects of the oxytocin receptor antagonists and restored the control delays (53.4 ± 1.7 min, $n = 18$; $P < 0.01$). These data indicate that maternal oxytocin exerts a neuroprotective action on fetal neurons during parturition and that this action is likely due to a reduction of $[Cl^-]_i$.

Our results suggest that oxytocin, in addition to its well-established role in labor and lactation and its multiple effects in the adult central nervous system (*19*–*25*), also exerts a powerful action on fetal neurons. This mechanism adds a previously unknown facet to the plasticity of GABA signaling via modulation of $[Cl^-]_i$ (*4*, *33*). The dual action produced by a single messenger in the mother and fetus enables a perfect timing for adaptation of fetal neurons to delivery.


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**Supporting Online Material**

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# ATP Release Guides Neutrophil Chemotaxis via P2Y2 and A3 Receptors


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**Cells must amplify external signals to orient and migrate in chemotactic gradient fields. We find that human neutrophils release adenosine triphosphate (ATP) from the leading edge of the cell surface to amplify chemotactic signals and direct cell orientation by feedback through P2Y2 nucleotide receptors. Neutrophils rapidly hydrolyze released ATP to adenosine that then acts via A3-type adenosine receptors, which are recruited to the leading edge, to promote cell migration. Thus, ATP release and autocrine feedback through P2Y2 and A3 receptors provide signal amplification, controlling gradient sensing and migration of neutrophils.**


Neutrophils are primary phagocytic cells with important roles in host defense and tissue repair. However, activated neutrophils damage host tissues and contribute to chronic inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and asthma (*1*). A key feature of neutrophils is their ability to detect and migrate to compromised tissues by following a concentration gradient of chemotactic substances released from microbial pathogens or injured cells.

Neutrophils can respond to chemoattractant gradients that differ in concentration by as little as 1% across the length of the cell body (*2*). Chemotaxis must involve signal amplification because a strongly polarized distribution of intracellular signal-transduction components is observed even in shallow gradients. The mechanisms of signal amplification are unclear (*3*, *4*). We identified the polarized release of adenosine triphosphate (ATP), the activation of P2Y2 receptors, and the translocation and activation of A3 adenosine receptors as key mechanisms of signal amplification that control cell orientation and direct the migration of neutrophils.

Membrane deformation caused by mechanical or osmotic stress induces the release of cellular ATP from mammalian cells; however, detailed information on the underlying mechanisms is lacking (*5*, *6*). Because cell migration also involves membrane deformation, we tested whether the stimulation of human neutrophils with the chemoattractant *N*-formyl-Met-Leu-Phe (FMLP) causes ATP release. Treatment of cells ($10^7$ cells in 250 μl of solution) with 100 nM FMLP rapidly tripled extracellular ATP concentrations in bulk media (Fig. 1, A and B) by inducing the release of ~0.5% of their ATP pool. Concentrations of extracellular ATP and its hydrolytic products adenosine monophosphate (AMP) and adenosine peaked 5 min after FMLP stimulation; but while the concentration of ATP returned to basal levels after 15 min, AMP and adenosine concentrations remained >5–fold above baseline (Fig. 1B), which is consistent with the presence of ecto–adenosine triphosphatases (ecto-ATPases) (*7*, *8*). Neutrophils completely hydrolyzed exogenous ATP (5 μM) within 2 min after ATP addition, which suggests that they have potent ecto-ATPase activity (Fig. 1C).

Because membrane deformation occurs predominantly at the leading edge closest to the chemoattractant source, we hypothesized this region to be the principal site of ATP release. Fluorescent microscopy that was used to visualize ATP release [based on conversion of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to its reduced form NADPH] revealed that neutrophils discharge ATP within seconds after FMLP stimulation (Fig. 1D and movies S1 to S3), with ATP release highest near the cell membrane with the greatest degree of protrusion (Fig. 1D, inset).

Extracellular ATP and adenosine modulate neutrophil functions, including chemotaxis (*8*, *9*). We tested the effect of apyrase, which hydrolyzes ATP, on chemotaxis in a trans-well system composed of upper wells with neutrophils and lower wells with 1 nM FMLP separated by a filter with 3-μm pores. Addition of apyrase to the upper wells reduced chemotaxis by nearly 100% (Fig. 2A).


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Apyrase also inhibited FMLP-induced superoxide formation, implying that FMLP-promoted responses require ATP. Addition of apyrase to the lower wells reduced chemotaxis only by ~40%, which suggests that ATP release is essential for the initiation of chemotaxis but not for maintaining migration once it is initiated. Microscopic analysis of neutrophil chemotaxis toward the tip of a micropipette filled with 100 nM FMLP confirmed this conclusion: Although 79% of control cells migrated toward FMLP with <60° angular deviation from a straight path, the addition of apyrase (10 U/ml) diminished the proper orientation of cells so that only 17% migrated correctly to the chemotactic source (Fig. 2B and movies S4 and S5). Other ATP-hydrolytic enzymes (e.g., ATPase and alkaline phosphatase) had similar effects (fig. S1, A and B), confirming ATP release to be crucial for gradient sensing and cell orientation.

Using the trans-well assay, we found that the nonhydrolyzable ATP analog adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S) increased FMLP-promoted chemotaxis and cell migration in the absence of FMLP, regardless of whether ATP-γ-S was added to the upper or lower well (Fig. 2B), which indicates that extracellular ATP induces chemokinesis (random cell migration) but is not chemotactic. Uniformly added ATP-γ-S (100 μM) impaired chemotaxis to a point source of FMLP, with only 31% of cells migrating along the correct path (Fig. 2C and movie S6), which implies that ATP-γ-S treatment obscures polarized ATP released from the cells and interferes with gradient sensing and proper cell orientation.

Adenosine and ATP are respective ligands of a family of four P1 adenosine (A1, A2a, A2b, and A3) and 15 P2 nucleotide receptors that include ionotropic P2X (P2X1-7) and G protein–coupled P2Y (P2Y1, 2, 4, 6, 11, 14) subtypes (*10*, *11*). We used the general P2-receptor antagonist suramin to test the role of P2 receptors in chemotaxis. Suramin reduced chemotaxis by 80% (Fig. 2D), whereas the P2X-selective antagonists 1-[*N*,*O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) and oxidized ATP did not (fig. S1C), implying that P2Y receptors are involved in neutrophil chemotaxis.

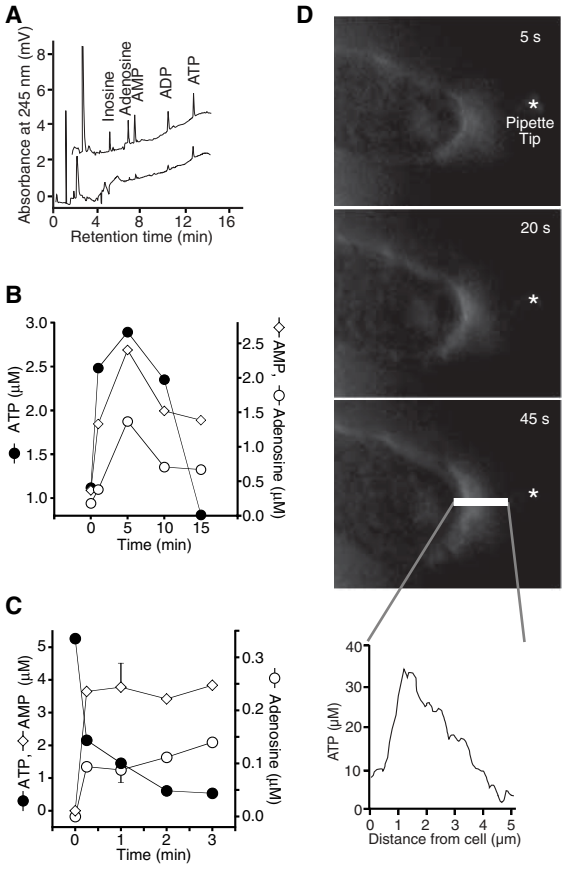


**Fig. 1.** Release of ATP by FMLP-stimulated neutrophils and formation of adenosine. (**A**) ATP released in response to FMLP was determined with high-performance liquid chromatography (HPLC) analysis of extracellular ATP and its breakdown products. A pair of chromatograms shows ATP and its hydrolytic products in the extracellular environment of neutrophils before (lower trace) or after (upper trace) stimulation with 100 nM FMLP. ADP, adenosine diphosphate. (**B**) Extracellular concentrations of ATP, AMP, and adenosine measured with HPLC analysis at different time points after stimulation of neutrophils with 100 nM FMLP. (**C**) Extracellular ATP, AMP, and adenosine at different times after addition of exogenous ATP to isolated neutrophils. Error bars indicate SD of triplicate determinations. (**D**) Release of ATP from living cells stimulated in a gradient field of FMLP generated with a micropipette (asterisks) was visualized with the use of a two-enzyme assay system that catalyzes the conversion of $NADP^+$ to NADPH in the presence of ATP. The generation of NADPH was monitored in real time by means of fluorescence microscopy (excitation wavelength, 340 nm; emission wavelength, 460 nm). Images were captured at 12 frames per minute. (**Inset**) Extracellular ATP concentrations based on calibration of gray values with a known ATP standard.

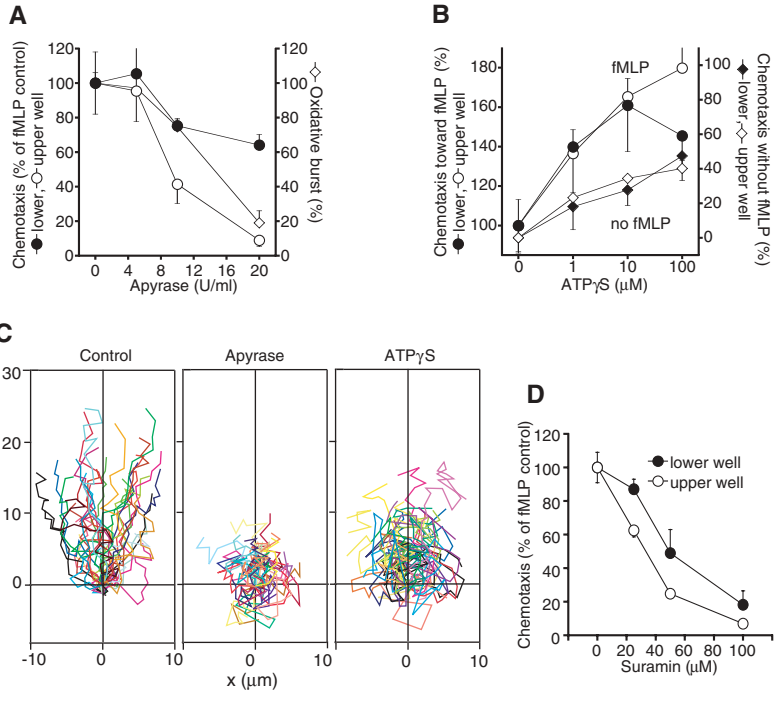


**Fig. 2.** Effect of exogenous ATP on neutrophil chemotaxis. Trans-well assays with neutrophils in upper wells separated from lower wells containing 1 nM FMLP by a filter with 3-μm pore size were used to assess chemotaxis. (**A**) Effect of apyrase added to the lower or upper well on neutrophil chemotaxis and on FMLP-induced oxidative burst. Cell responses are expressed as a percent of the response to FMLP in the absence of apyrase. (**B**) Treatment with ATP-γ-S in the presence (circles) or absence (diamonds) of FMLP. (**C**) Cell migration studied under the microscope was analyzed by tracing the paths of cells migrating toward a micropipette tip containing 100 nM FMLP in the absence (left) or presence (middle) of 10 U/ml of apyrase or 100 μM ATP-γ-S (right). The *y* axis of the traces represents the direction toward the chemoattractant source, and the *x* axis shows the deviation from the straight path. Cell traces were arranged to show their origins at $x = y = 0$. (**D**) Effect of the P2-receptor antagonist suramin on FMLP-induced cell migration. Error bars in (A), (B), and (D) indicate SD of triplicate determinations.

Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis suggests that neutrophils and human promyelocytic HL60 cells express predominantly A2a-, P2Y2-, and A3-receptor–derived mRNA (Fig. 3, A and B). Immunostaining showed that A3 receptors, but not A2a or P2Y2 receptors, are concentrated at the leading edge of polarized cells (fig. S2, A and B), which suggests that A3 receptors may be involved in chemotaxis. In the trans-well assay, adenosine (1 nM to 1 μM) enhanced chemotaxis, particularly when added in the lower wells along with FMLP (Fig. 4A). Higher concentrations of exogenous adenosine slightly diminished chemotaxis (Fig. 4A and fig. S3A), likely by either activating A2a receptors, which are widely recognized for their inhibitory effects on neutrophils (*8*–*12*) or obscuring endogenous adenosine generated at the leading edge of migrating cells. Removal of extracellular adenosine with adenosine deaminase (ADA), which converts adenosine to inosine, or inhibition of A3 receptors with 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191) inhibited chemotaxis toward FMLP (Fig. 4, B and E), platelet-activating factor, 3% autologous zymosan-activated serum, interleukin-8, and live bacteria (fig. S3, B to D). Inosine generated by ADA did not affect chemotaxis (fig. S3E).

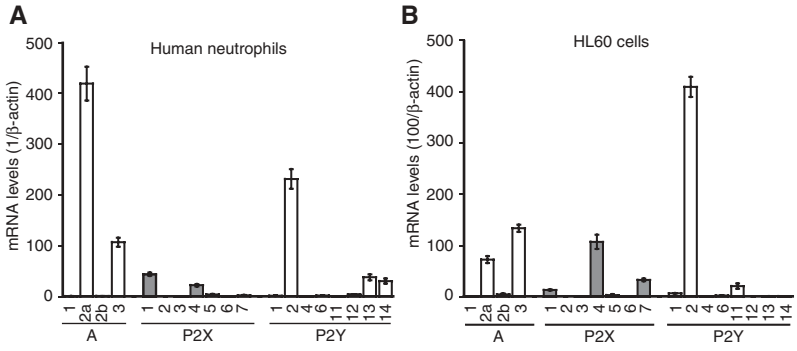


**Fig. 3.** P1- and P2-receptor expression in neutrophils (**A**) and HL60 cells (**B**). P1- and P2-receptor mRNA expression in human neutrophils and HL60 cells was estimated with real-time RT-PCR analysis and expressed in relation to β-actin. Error bars in (A) and (B) indicate SD of triplicate determinations.

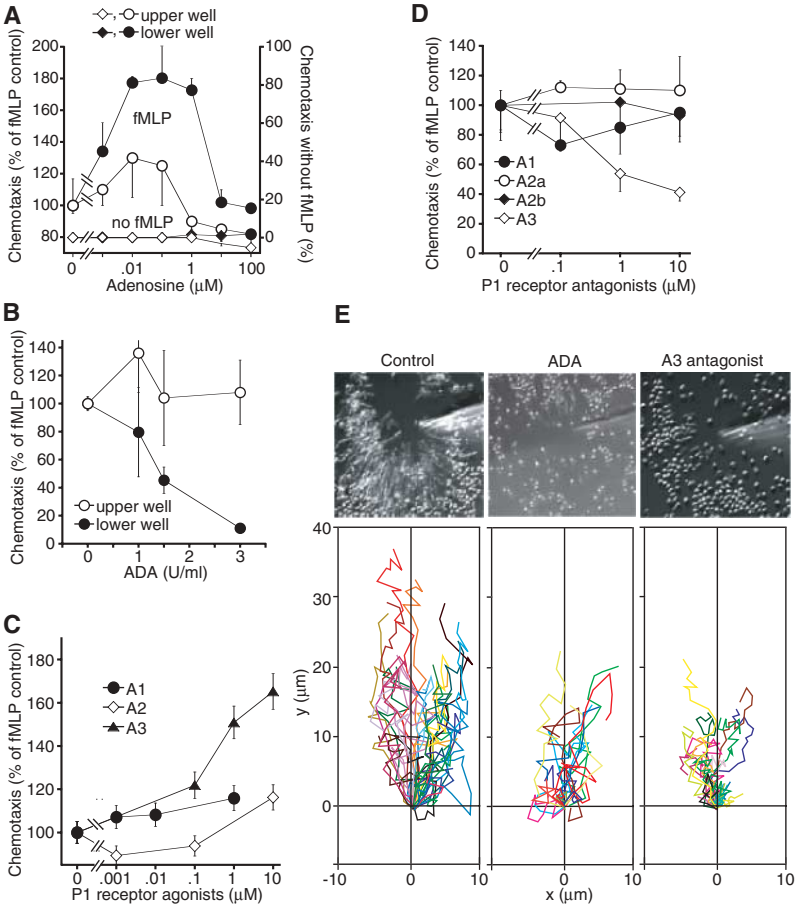


**Fig. 4.** Role of adenosine and P1 receptors in neutrophil migration. (**A**) The effect of exogenous adenosine added to the lower or upper wells on neutrophil chemotaxis was assessed with the trans-well assay in the presence (circles) or absence (diamonds) of FMLP. (**B**) Effect of ADA on FMLP-induced chemotaxis. (**C**) Effects of the A3-receptor–selective agonist *N*(6)-(3-iodobenzyl) adenosine-5'-*N*-methylcarboxamide (IB-MECA) and of the A2- and A1-receptor–selective agonists 2-*p*-[2-carboxyethyl] phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride (CGS 21680) and *N*6-cyclopentyl-adenosine (CPA), respectively, on chemotaxis toward FMLP. (**D**) Effects of A3-receptor–selective antagonist MRS 1191 and antagonists of other P1 receptors on chemotaxis toward FMLP. (**E**) Composite images and cell migration traces of cells migrating toward a micropipette tip containing 100 nM FMLP in the absence or presence of 10 U/ml of ADA or 10 μM MRS 1191. Error bars in (A) to (D) indicate SD of triplicate determinations.

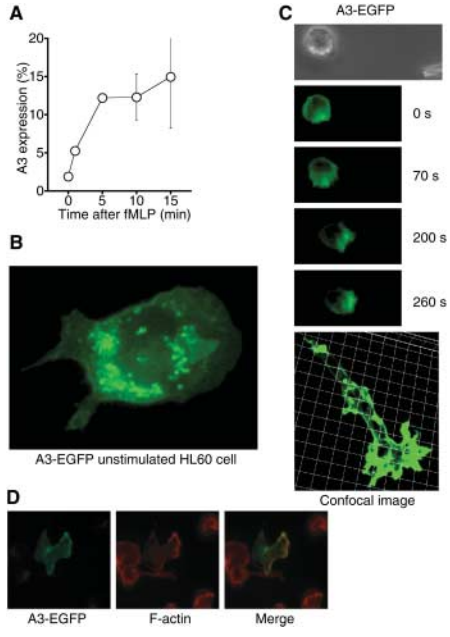


**Fig. 5.** Localization of A3 receptors to the leading edge of migrating cells. (**A**) The cell surface expression of A3 receptors of human neutrophils at different time points after stimulation of cells with 100 nM FMLP was assessed with flow cytometry and a primary antibody recognizing an extracellular domain of the receptor. Error bars indicate SD of triplicate determinations. (**B**) Fluorescent image of an unstimulated HL60 cell expressing an A3-EGFP fusion protein. (**C**) HL60 cell expressing an A3-EGFP fusion protein migrating toward a micropipette tip containing 100 nM FMLP (bright field image on top). The confocal image at the bottom shows an HL60 cell migrating from the top left to the bottom right corner. (**D**) Colocalization of A3-EGFP fusion protein and actin in cells globally stimulated with 100 nM FMLP.

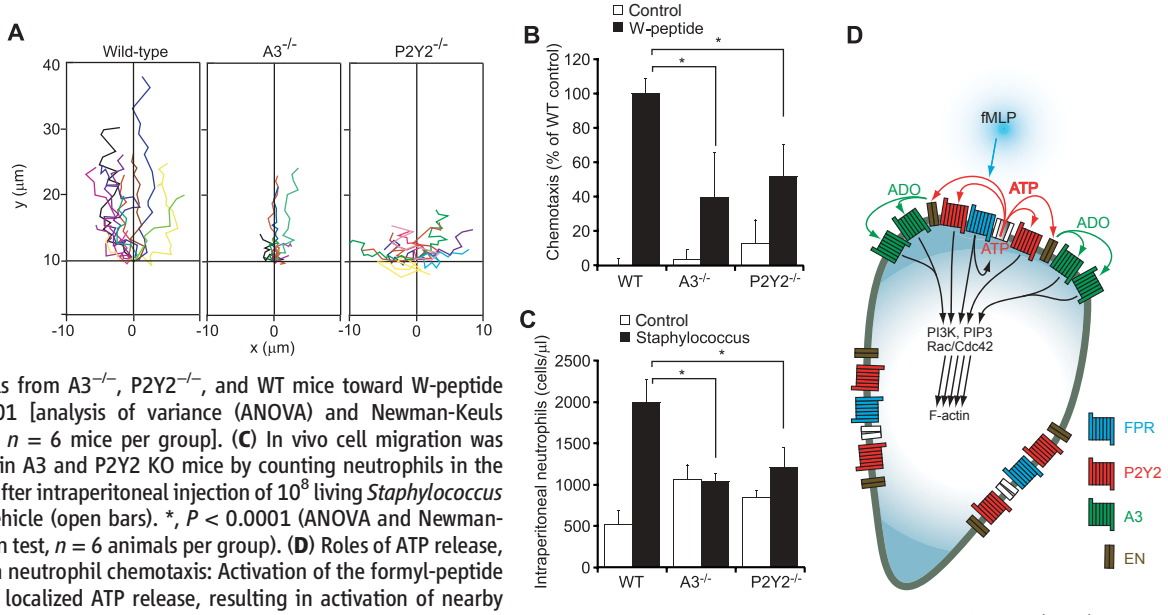


**Fig. 6.** P2Y2 and A3 receptors control neutrophil chemotaxis in vitro and in vivo. (**A**) Migration paths of neutrophils [isolated from the bone marrow of wild-type (WT) mice and mice deficient of A3 and P2Y2 receptors] migrating toward the FMLP-receptor ligand W-peptide (100 nM). (**B**) Trans-well chemotaxis assays of neutrophils from $A3^{-/-}$, $P2Y2^{-/-}$, and WT mice toward W-peptide (100 nM). *, $P < 0.0001$ [analysis of variance (ANOVA) and Newman-Keuls multiple-comparison test, $n = 6$ mice per group]. (**C**) In vivo cell migration was assessed in WT mice and in A3 and P2Y2 KO mice by counting neutrophils in the peritoneal cavity 4 hours after intraperitoneal injection of $10^8$ living *Staphylococcus* bacteria (solid bars) or vehicle (open bars). *, $P < 0.0001$ (ANOVA and Newman-Keuls multiple-comparison test, $n = 6$ animals per group). (**D**) Roles of ATP release, P2Y2, and A3 receptors in neutrophil chemotaxis: Activation of the formyl-peptide receptor (FPR) stimulates localized ATP release, resulting in activation of nearby P2Y2 receptors that amplify chemotactic signals and gradient sensing by stimulating the production of phosphoinositide 3-kinase (PI3K), phosphatidylinositol 3,4,5-trisphosphate (PIP3), and recruitment of Rac, Cdc42, and F-actin to the leading edge. Translocation of A3 receptors to the leading edge, adenosine (ADO) formation by ecto-ATPases/nucleotidases (EN), and autocrine activation of A3 receptors facilitate directed migration. Error bars in (B) and (C) indicate SD.

The finding that the agonists and the antagonist of A3 receptors (but not of other P1-receptor subtypes) affected neutrophil chemotaxis further suggested a special role for A3 receptors in chemotaxis (Fig. 4, C and D). To determine whether A3 receptors control gradient sensing or migration speed, we examined the effect of MRS 1191 on neutrophil chemotaxis under the microscope. MRS 1191 (10 μM) reduced the migration speed of cells toward FMLP from 2.4 ± 1.0 to 0.5 ± 0.7 μm/min (Fig. 4E and movies S7 and S8), whereas gradient sensing seemed largely unaffected. Thus, A3 receptors appear to be the key adenosine receptors that regulate neutrophil chemotaxis by controlling cell migration.

Flow cytometric analysis revealed that FMLP stimulation rapidly increases surface expression of A3 receptors in human neutrophils (Fig. 5A). This finding was confirmed with HL60 cells stably transfected with an A3 receptor–enhanced green fluorescent protein (EGFP) construct and differentiated with dimethyl sulfoxide to induce a neutrophil-like phenotype capable of chemotaxis (*13*, *14*). A3 receptors in resting cells were predominantly localized in cytosolic granules (Fig. 5B). Upon exposure to an FMLP gradient, A3 receptors, but not P2Y2 receptors, rapidly translocated to the cell surface near the leading edge, accumulated on filopodia-like protrusions, and colocalized with F-actin (Fig. 5C and movies S9 and S10), a marker for the leading edge of polarized cells (*15*).

We further studied the chemotaxis of neutrophils from bone marrow of A3- and P2Y2-receptor knockout (KO) mice ($A3^{-/-}$ and $P2Y2^{-/-}$) using the murine chemotactic peptide Trp-Lys-Tyr-Met-Val-Met-$NH_2$ (W-peptide) (*16*). Chemotaxis of neutrophils from both KO mouse strains was less than that of wild-type mice (Fig. 6, A and B). Neutrophils lacking A3 receptors showed correct directionality but diminished speed, whereas cells without P2Y2 receptors showed a loss in gradient sensing. We confirmed the importance of both receptors for neutrophil chemotaxis in vivo by assessing cell recruitment to the peritoneal cavity of KO mice intraperitoneally injected with 1 nM W-peptide or $10^8$ *Staphylococcus aureus* bacteria (Fig. 6C).

Our results lead to a model in which polarized ATP release, in response to chemotactic receptor stimulation, facilitates gradient sensing by activating multiple adjacent P2Y2 receptors that control cell orientation in a chemotactic gradient. Adenosine formation and A3-receptor accumulation at the leading edge likely constitute a second stage of autocrine signal amplification that facilitates chemotaxis by controlling migration speed (Fig. 6D). Other receptors, such as A2a receptors, may contribute to chemotaxis by facilitating membrane retraction at the receding end. This system may help neutrophils locate bacteria and damaged tissues, which also generate extracellular ATP (*17*). In view of their importance for cell migration, P2Y2 and A3 receptors are potential therapeutic targets for the treatment of inflammatory diseases (*18*, *19*).

Chemotaxis in *Dictyostelium discoideum* is controlled by extracellular signaling systems that use cyclic AMP (cAMP) production and the activation of cAMP receptors (*20*–*22*). The results shown here define an analogous mammalian navigation system based on polarized ATP release and purinergic receptor activation in neutrophils.

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## Supporting Online Material

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