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Activation of the A_3 adenosine receptor inhibits fMLP-induced Rac activation in mouse bone marrow neutrophils

Dharini van der Hoeven, Elizabeth T. Gizewski, John A. Auchampach *

Department of Pharmacology and Toxicology and the Cardiovascular Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, United States

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ABSTRACT

Adenosine is released from injured or hypoxic tissues where it exerts numerous anti-inflammatory effects including suppression of neutrophil functions. Although most previous work has implicated the A_{2A}AR, we have recently shown that selective activation of the abundantly expressed A_3AR inhibits neutrophil superoxide production and chemotaxis providing a potential mechanistic explanation for the efficacy of A_3AR agonists in experimental animal models of inflammation. In this study, we hypothesized that the A_3AR suppresses neutrophil functions by inhibiting the monomeric GTPase Rac, a central regulator of chemokine-directed neutrophil migration and superoxide production. We found that pre-treating neutrophils with the highly selective A_3AR agonist CP-532,903 reduced fMLP-induced Rac activation using an ELISA-based assay that detects all three Rac isoforms. CP-532,903 also inhibited fMLP-induced F-actin formation, a downstream effector function of Rac relevant to neutrophil migration, but not activation of ERK1/2 or p38. Pre-treating neutrophils with CP-532,903 did not stimulate cAMP production or alter fMLP-induced calcium transients, implicating that A_3AR stimulation does not inhibit Rac activation or neutrophil activities by suppressing Ca^{2+} signaling, elevating the intracellular concentration of cAMP, or by cross-desensitizing fMLP receptors. Our results suggest that activation of the A_3AR signals to suppress neutrophil functions by interfering with the monomeric GTPase Rac, thus contributing to the ant-inflammatory actions of adenosine.

1. Introduction

Neutrophils are attracted to inflamed tissues through the production of chemoattractant mediators including chemokines, lipid molecules (platelet-activating factor, leukotrienes), complement components (C5a), and bacterial proteins including N-formylated peptides [1,2]. Most of these molecules bind to cell surface G protein-coupled receptors that stimulate neutrophils to migrate, and ultimately to phagocytose microorganisms and cell debris, secrete granule contents containing degradative enzymes, activate the NADPH oxidase to generate reactive oxygen species, and stimulate the synthesis of other pro-inflammatory molecules that help to recruit additional immune cell populations [1,2]. While these actions of neutrophils are critical for normal wound healing, the excessive release of toxic mediators can damage host tissue contributing to the pathogenesis of numerous acute and chronic inflammatory diseases.

Adenosine is formed in inflamed tissues from the enzymatic degradation of ATP released from activated or injured cells, which serves to dampen the inflammatory reaction and promote inflammation resolution by suppressing the activity of most cells of the immune system including the neutrophil [3,4]. Adenosine potently inhibits neutrophil adhesion to endothelial cells, degranulation, superoxide production, and pro-inflammatory mediator production [3,4]. Among the four adenosine receptor (AR) subtypes (A_1, A_{2A}, A_{2B}, and A_3), most previous studies have implicated the A_{2A}AR in mediating the inhibitory effects of adenosine on neutrophils via the cAMP/protein kinase A (PKA) signaling axis and/or through cAMP-independent activation of a protein phosphatase [3,4]. However, we have recently discovered that activating the G_i protein-coupled A_3AR also functions in murine neutrophils to inhibit superoxide production and chemotaxis [5]. Suppression of neutrophil activity represents a potential mechanism by which A_3AR agonists provide benefit in experimental animal models of inflammation [6–11].

The small GTPase Rac plays a central role in regulating responses to inflammatory signals in neutrophils. Rac2 is a

Abbreviations: ADA, adenosine deaminase; AR, adenosine receptor; C5a, complement component 5a; CGS 21680, 2-[p-(2-carboxyethyl)phenethylamino]-5′-N-ethylcarboxamidoadenosine; CP-532,903, (2S,3S,4R,5R)-3-amino-5-[6-(2,5-dichlorobenzylamino)purin-9-yl]-4-hydroxytetrahydrofuran-2-carboxylic acid methylamide; fMLP, formylated-methionine-leucine-phenylalanine; GEF, guanine nucleotide exchange factor; KO, knockout; WT, wild-type.

* Corresponding author at: Department of Pharmacology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, United States. Tel.: +1 414 456 5643; fax: +1 414 456 6545.
E-mail address: jauchamp@mcw.edu (J.A. Auchampach).

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necessary component of the NADPH oxidase complex that is assembled in endosomes and at the plasma surface upon chemoattractant receptor stimulation [12]. Rac2 also regulates rearrangement of the cytoskeleton and neutrophil migration [13–15]. Requirement of Rac2 in chemotaxis and the formation of reactive oxygen species has been demonstrated in studies using neutrophils from Rac2-null mice [14] and from patients that carry key Rac2 mutations [16,17]. Although Rac2 is the primary isoform found in human hematopoietic cells, Rac1 is equally expressed in murine neutrophils where it also regulates both superoxide production and direction sensing during chemotaxis [18,19].

In this study, we provide evidence that the A_3AR inhibits fMLP-induced Rac activation in murine neutrophils. This occurs by mechanisms that do not involve alterations in Ca^{2+} signaling, cAMP elevation, or cross-desensitization of the fMLP receptor. Suppression of chemokine receptor-induced activation of Rac represents a potential intracellular signaling mechanism by which the A_3AR suppresses neutrophil activities.

2. Materials and methods

2.1. Materials

Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Fura2-AM and pluronic F-127 were purchased from Molecular Probes–Invitrogen (Eugene, OR). Anti-phospho-ERK1/2 (extracellular signal-regulated kinase 1/2), anti-phospho-p38, anti-ERK1/2, anti-p38 and horseradish peroxidase-linked anti-rabbit IgG were purchased from Cell Signaling Technology, Inc. (Danvers, MA). CL-XPosure™ Film and Restore Western Blot stripping buffer were from Pierce Biotechnology, Inc. (Rockford, IL). cAMP assay kits and Hybond™-C nitrocellulose membrane were obtained from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). Ro 20-1724 was obtained from BIOMOL International L.P. (Plymouth Meeting, PA). Western Lighting™ Chemiluminescence Reagent Plus was from PerkinElmer LAS, Inc. (Boston, MA). G-LISA Kit was purchased from Cytoskeleton, Inc. (Denver, CO). Percoll was purchased from Amersham Biosciences (Piscataway, NJ). CP-532,903 was a gift from Dr. W. Ross Tracey (Pfizer Global Research and Development, Groton, CT), adenosine deaminase (ADA) was purchased from Roche Applied Science (Indianapolis, IN), and all remaining drugs and reagents were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Mice

C57BL/6 wild-type (WT) mice were purchased from Taconic Farms (Germantown, NY). Congenic C57BL/6 A_3KO mice were a kind gift from Dr. Marlene Jacobson (Merck Research Labs, West Point, PA [20]). All animal experiments were conducted with approval of the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

2.3. Isolation of mouse bone marrow neutrophils

Morphologically mature neutrophils were purified from mouse bone marrow by isotonic Percoll gradient centrifugation, as previously described [5,21]. Briefly, mice were euthanized by anoxia with carbon dioxide. Tibias and femurs of mice were flushed with neutrophil isolation buffer (1× HBSS without Ca^{2+} and Mg^{2+}, and containing 0.4% sodium citrate) and layered on a three-step Percoll gradient (72%, 64% and 52%). Following centrifugation at 1060 × *g* for 30 min, cells at the 72%:64% interface, were removed and washed once with isolation buffer before use in experiments.

2.4. Rac activation assays

Freshly isolated neutrophils were re-suspended in HBSS containing 1 unit/mL ADA and then aliquoted into Eppendorf tubes (2.5×10^6 cells/250 μL). After incubating at 37 °C for 30 min in the presence of vehicle or CP-532,903, the cells were stimulated with fMLP (1 μM) for the times indicated. The reactions were terminated by the addition of two volumes of ice-cold neutrophil isolation buffer with simultaneous quick chilling in a dry ice/ethanol bath. Total activated Rac (isoforms 1, 2 and 3) in the cell lysates (1 μg/μL) was quantified using the G-LISA kit that utilizes 96-well plates coated with a Rac-GTP binding domain-containing effector protein and a non-specific Rac antibody. In control assays, the cells were first subjected to one freeze–thaw cycle after which the lysates were incubated for 10 min at 37 °C in the reaction buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, and 6 mM $MgCl_2$, pH 7.4) containing 200 μM GTPγS, prior to assay for activated Rac.

2.5. F-actin measurements

Freshly isolated neutrophils were re-suspended in HBSS buffer containing 1 unit/mL ADA and aliquoted into Eppendorf tubes (2×10^6 cells/250 μL). After incubating for 30 min at 37 °C in the presence of vehicle or CP-532,903, the cells were stimulated with fMLP (1 μM) for 30 s, after which the cells were fixed with 3.7% paraformaldehyde (in HBSS without Ca^{2+}/Mg^{2+}) for 30 min on ice. The cells were washed and then incubated for another 30 min on ice with FITC-phalloidin (2.5 μM) in PBS/2% FBS supplemented with 100 μg/mL L-α-palmitoyl-lysophophatidylcholine to permeabilize and stain the cells. F-actin content was quantified by flow cytometry using a Becton Dickinson FACSCaliber flow cyctometer by measuring a total of at least 10,000 cells per sample.

2.6. Western blot analysis for phosphorylation of ERK1/2 and p38

Freshly isolated neutrophils were re-suspended in HBSS buffer containing 1 unit/mL ADA and aliquoted into Eppendorf tubes (~2.5×10^6 cells/250 μL). After incubating for 30 min at 37 °C with vehicle or CP-532,903, the cells were stimulated with fMLP (1 μM) for the times indicated. The assays were terminated by the addition of two-fold volume of ice-cold neutrophil isolation buffer followed by rapid chilling in dry ice/ethanol. Cell lysates were prepared by adding 50 μL of Triton-X lysis buffer consisting of 25 mM Tris–HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton-X-100, 40 mM β-glycerophosphate, 40 mM para-nitrophenylphosphate, 1 mM sodium orthovanadate, 1 mM phenyl ethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 10 μg/mL aprotinin. The lysates were clarified by centrifugation at 16,000 × *g* for 5 min at 4 °C and the protein concentrations of the soluble extracts were quantified using the BioRad (Bradford) protein assay. The extracts (20 μg protein) were denatured with 5× Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 20% β-mercaptoethanol, and 0.0025% bromophenol blue) and boiled for 5 min prior to separation by standard 10% SDS-polyacrylamide gel electrophoresis. Proteins were wet blotted onto nitrocellulose membranes and probed with anti-phospho-ERK1/2 (1:2000) or anti-phospho-p38 (1:2000) antibodies. The blots were stripped with Restore PLUS Western blot stripping buffer (Pierce) and reprobed with antibodies against the respective total proteins (anti-ERK1/2, 1:2000; anti-p38, 1:2000). Horseradish peroxidase-conjugated secondary antibodies (1:1000) were visualized by enhanced chemiluminescence detection (Western Lightning™ Reagent Plus, PerkinElmer). Densitometry analysis was performed using Scion Image software (from the National Institutes of Health).

2.7. Intracellular Ca^{2+} assays

Freshly isolated neutrophils were loaded with the Ca^{2+}-specific fluorescent probe Fura-2 AM (5 μM) in neutrophil isolation buffer for 30 min at 37 °C. Cells were washed and re-suspended in HBSS containing 1 unit/mL ADA at a concentration of 1×10^6 cells/mL. Fluorescence at baseline and after the addition of various activating agents was continuously measured with a spectrofluorimeter (Becton Dickinson) in a stirred thermostable cuvette (37 °C) using an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm. The intracellular concentration of calcium ($[Ca^{2+}]_i$) was calculated using the Grynkiewicz equation [22]:

$$[Ca^{2+}]_i = \frac{K_d Q(R - R_{min})}{R_{max} - R}$$

where $[Ca^{2+}]_i$ is given in nM units, K_d is the dissociation constant of Fura-2 AM (224 nM under standard conditions), R represents the ratio of fluorescence (F) emission at 510 nm following excitation at 340 and 380 nm (F340/F380) and Q is the ratio of minimal and maximal fluorescence following excitation at 380 nm. R_{max} was measured after the addition of 20 μM digitonin to release all of the intracellular FURA-2 AM. R_{min} was subsequently measured after the addition of 50 mM EGTA to chelate the free calcium. All results are plotted as $[Ca^{2+}]_i$ (nM) versus time.

2.8. Quantification of intracellular cAMP accumulation

Freshly isolated neutrophils were re-suspended in HBSS containing 1 unit/mL ADA and 20 μM Ro 20-1724 (phosphodiesterase inhibitor) and then transferred to polypropylene tubes (1×10^5 cells/200 μL). After equilibrating at 37 °C for 5 min, vehicle or agonists were added at the concentrations indicated for 15 min. The assays were terminated by adding 500 μl of 0.15N HCl. cAMP in the acid extract was determined by radioimmunoassay according to the manufacturer's protocol (GE Healthcare; Piscastaway, NJ).

3. Results

3.1. Activation of the A_3AR reduces fMLP-induced Rac activation

We have previously observed that activating the A_3AR inhibits superoxide production and chemotaxis of mouse bone marrow neutrophils in response to fMLP as well as a panel of other chemoattractive agents [5]. Considering that Rac plays an important role in regulating both of these responses in murine neutrophils, we examined whether stimulating the A_3AR interferes with fMLP-induced Rac activation using an ELISA-based assay that quantifies the active guanosine-5′-triphosphate (GTP)-bound form of all three Rac isoforms. Like other small GTPases, Rac cycles between a GDP-bound inactive state and a GTP-bound active state. Initially, we examined the time-course of Rac activation in response to 1 μM fMLP. As shown in Fig. 1, the level of active Rac was increased as early as 5 s after exposure to fMLP and returned to baseline levels by 1 min. In positive control assays, active Rac was increased nearly four-fold when cell lysates were treated with the non-hydrolyzable GTP analog GTPγS (Fig. 1A). Previous studies using human and murine neutrophils have similarly reported that Rac is rapidly and transiently activated in response to fMLP [23,24]. The magnitude and time-course of fMLP-induced activation was similar in assays using neutrophils isolated from A_3KO mice (Fig. 1A).

We subsequently examined whether treating neutrophils for 30 min with vehicle or the A_3AR agonist CP-532,903 (100 nM) reduces fMLP-induced Rac activation. A 30-min pretreatment

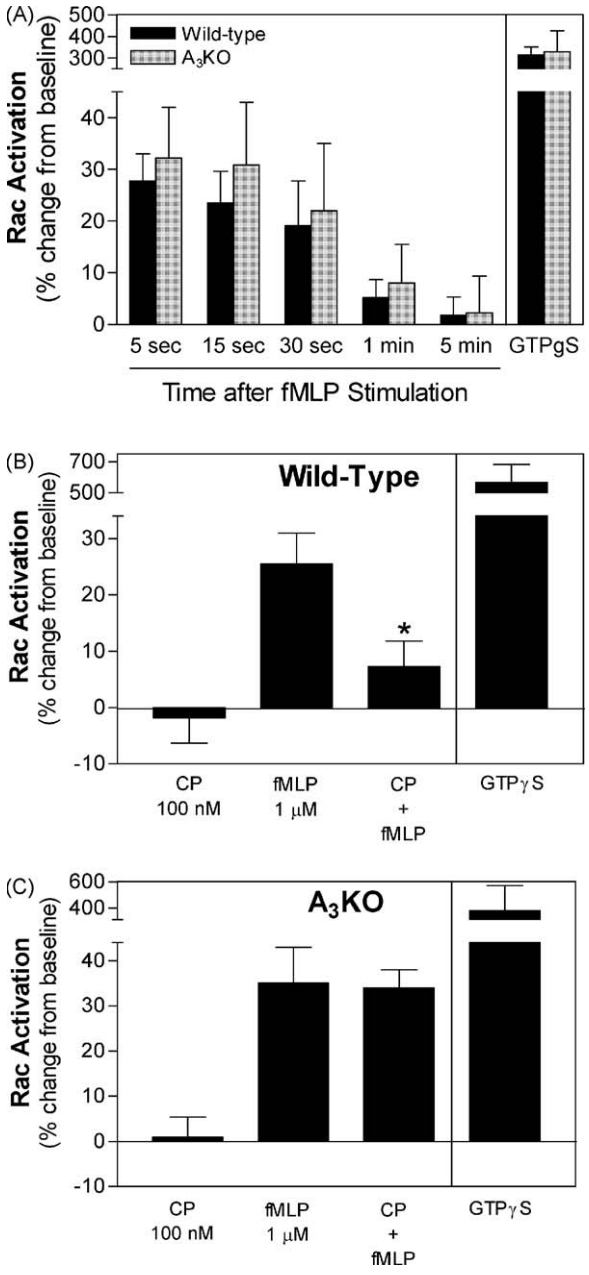

Fig. 1. Effect of CP-532,903 on fMLP-induced Rac activation in mouse bone marrow neutrophils obtained from wild-type mice and from A_3ARKO mice. Rac activity in whole-cell lysates was quantified using an ELISA-based assay kit (G-LISA kit, Cytoskeleton, Inc.), as described under Section 2. (A) The time-course of Rac activation following stimulation with fMLP (1 μM). Rac activity 15 s after the addition of 1 μM fMLP to wild-type (B) and A_3KO (C) cells pre-treated for 30 min with 100 nM CP-532,903 (CP). The data are presented as the percent increase over baseline activity. In control assays, cell lysates were incubated with 200 μM GTPγS. All assays were conducted in the presence of 1 unit/mL ADA. Mean ± SEM. $^*p < 0.05$ versus the fMLP-treated group by one-way ANOVA and Bonferroni's t-test, $n = 3$–7.

protocol was used since we observed previously that maximal inhibition of fMLP-induced superoxide production is achieved when the cells are exposed to CP-532,903 for at least 18 min [5]. Although treatment with CP-532,903 did not alter the basal level of active Rac, it markedly reduced the degree of activation produced by fMLP (Fig. 1B). This result indicates that stimulating the A_3AR in murine neutrophils inhibits the ability of fMLP receptors to couple to Rac signaling. To confirm that CP-532,903 functions specifically through activation of the A_3AR, parallel studies were conducted using neutrophils isolated from A_3KO mice [20]. As shown in

Fig. 1C, the inhibitory effect of CP-532,903 on fMLP-induced Rac activation was not apparent in assays using A_3KO neutrophils.

3.2. Activation of the A_3AR inhibits fMLP-induced F-actin generation but not fMLP-induced phosphorylation of ERK or p38

Both Rac1 and Rac2 participate in chemoattractant-induced neutrophil migration by promoting actin polymerization at the leading edge [15]. Rac2 has also been reported to be upstream of ERK1/2 and p38 mitogen-activated protein (MAP) kinase activation by chemoattractants [15], which importantly regulate neutrophil superoxide production and chemotaxis. We therefore examined whether activation of the A_3AR interferes with these two downstream effector functions of Rac. For F-actin assays, neutrophils in suspension were pre-treated with either vehicle or CP-532,903 (100 nM) for 30 min and then stimulated with fMLP (1 μM) for 30 s before staining the cells with FITC-conjugated phalloidin. For the MAP kinase assays, mouse bone marrow neutrophils were pre-treated with vehicle or CP-532,903 for 30 min and then stimulated with fMLP for up to 10 min after which phosphorylated ERK1/2 and p38 were quantified by Western immunoblotting. As shown in Fig. 2A, exposure to fMLP significantly increased F-actin content over two-fold in vehicle-treated control cells; this increase was nearly abolished in cells pre-treated with CP-532,903. Treatment with fMLP also induced rapid but transient phosphorylation of both ERK1/2 and p38 (Fig. 3). In contrast to the results of the F-actin assays, treatment with CP-532,903 did not inhibit fMLP-induced phosphorylation of either ERK1/2 or p38 kinases.

3.3. Activation of the A_3AR does not alter fMLP-induced intracellular Ca^{2+} transients or stimulate cAMP elevation

Activation of chemoattractant receptors including fMLP receptors induces transient elevations in intracellular Ca^{2+} [25,26], which is prerequisite for the pro-inflammatory activities of neutrophils including superoxide production and degranulation as well as adhesion required for cell migration [25,26]. It has previously been suggested that intracellular Ca^{2+} signaling may contribute to Rac activation in response to stimulation by G protein-coupled receptors [27]. Moreover, one potential mechanism by which A_{2A}AR activation inhibits the pro-inflammatory activity of neutrophils is by accelerating the sequestration of intracellular Ca^{2+} through elevation of cAMP and activation of protein kinase A (PKA; [26,28]). We therefore examined whether activation of the A_3AR influences fMLP-induced Ca^{2+} transients in murine neutrophils or stimulates cAMP production. As shown, treating cells with CP-532,903 had no effect on fMLP-induced Ca^{2+} transients (Fig. 4). In addition, treatment with CP-532,903 did not promote cAMP accumulation in neutrophils at concentrations as

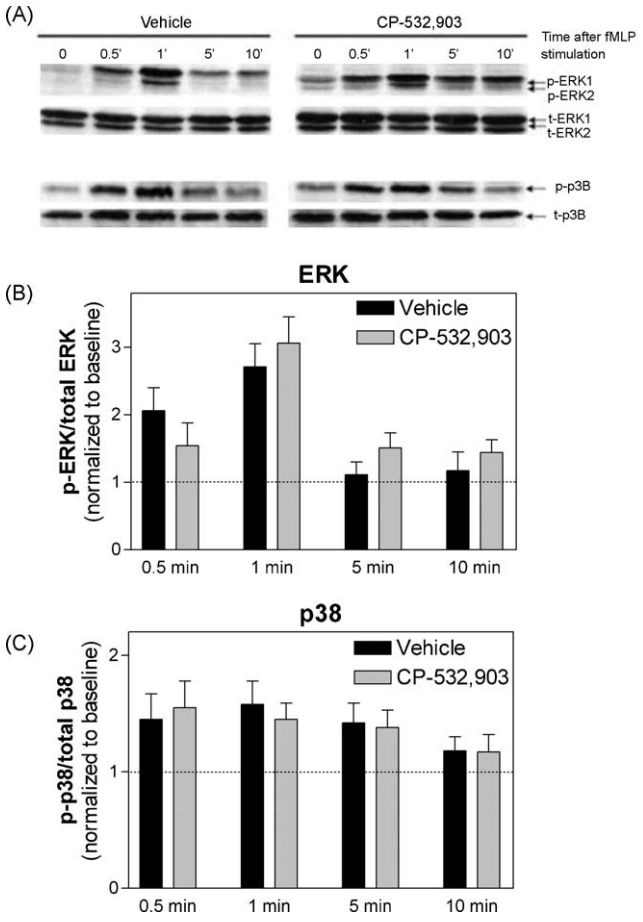

Fig. 3. Effect of CP-532,903 on fMLP-induced activation of ERK1/2 and p38 in mouse bone marrow neutrophils. (A) Representative Western immunoblots showing phosphorylated and total levels of ERK1/2 and p38. (B) and (C) show the results of densitometric analysis of the Western immunoblots for ERK1/2 and p38, respectively. Ratios of phosphorylated to total protein were normalized to baseline levels. Mean ± SEM. n = 3–4.

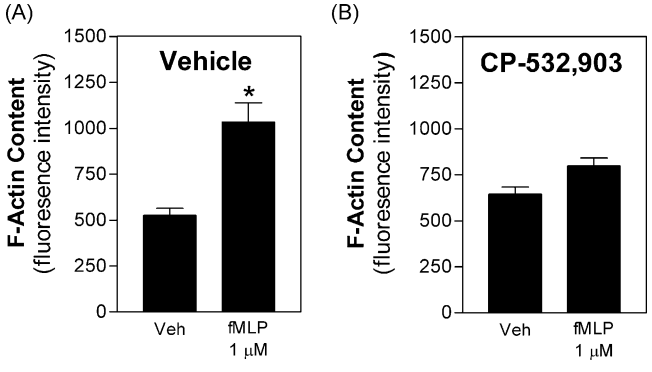

Fig. 2. Effect of CP-532,903 on fMLP-induced F-actin formation in mouse bone marrow neutrophils. Neutrophils were pre-treated with vehicle (A) or 100 nM CP-532,903 (B) for 30 min at 37 °C in the presence of 1 unit/mL ADA, and then stimulated with 1 μM fMLP for 30 s. Cells were stained with FITC-conjugated phalloidin and intracellular fluorescence was quantified by flow cytometry assessing a total of 10,000 cells per sample. Mean ± SEM. $^*p < 0.05$ versus the vehicle-treated group by Student's t-test, n = 8.

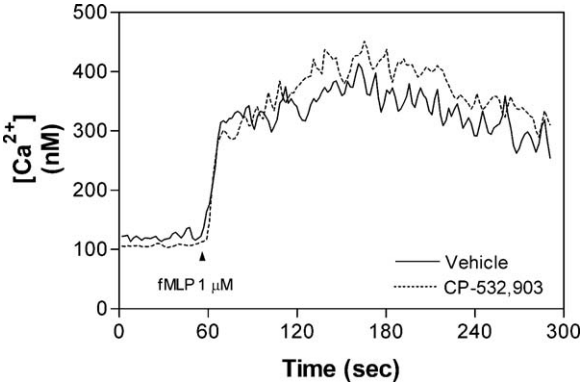

Fig. 4. Changes in intracellular [Ca^{2+}] in mouse bone marrow neutrophils in response to fMLP. The cells were pre-treated for 30 min with vehicle or CP-532,903 (100 nM) prior to measurement of intracellular [Ca^{2+}] during stimulation with fMLP (1 μM). The intracellular [Ca^{2+}] was measured in suspended cells loaded with FURA-2/AM in HBSS containing 1 unit/mL ADA, as described in Section 2. The data shown are representative of three to four independent experiments.

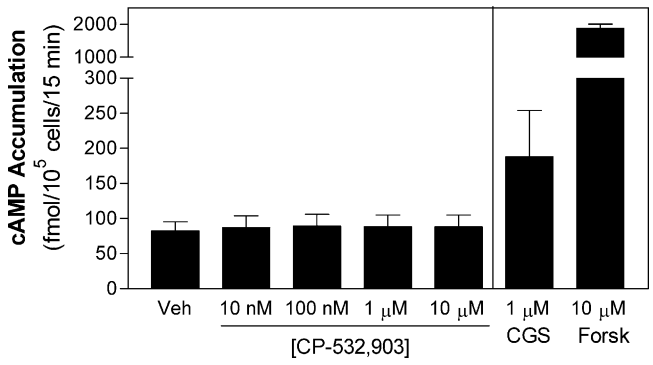

Fig. 5. CP-532,903 does not stimulate cAMP production in mouse bone marrow neutrophils. Neutrophils suspended in HBSS containing 1 unit/mL ADA and 20 μM Ro 20-1724 were stimulated with vehicle or increasing concentrations of CP-532,903 for 15 min. In control experiments, the cells were stimulated with the A_{2A}AR agonist CGS 21680 (1 μM) or forskolin (10 μM). The assays were terminated by adding 0.15N HCl. cAMP in the acid extract was determined by radioimmunoassay. Mean ± SEM. $^*p < 0.05$ versus the vehicle-treated group by one-way ANOVA and Dunnett's *t*-test, $n = 3$.

high as 10 μM (Fig. 5). In contrast, in control studies stimulating the cells with the A_{2A}AR agonist CGS 21680 or forskolin produced a significant increase in cAMP accumulation (Fig. 5). These results therefore indicate that activation of the A_3AR does not alter Ca^{2+} signaling responses in murine neutrophils. By inference, these results also demonstrate that activation of the A_3AR does not produce heterologous desensitization of fMLP receptors [29–33].

4. Discussion

We recently identified that the A_3AR is abundantly expressed in murine neutrophils and that activation of this AR subtype, along with the A_{2A}AR, inhibits fMLP-induced superoxide production and also slows chemotaxis [5]. Considering that it couples to G_i inhibitory proteins similar to most chemoattractant receptors, the intracellular mechanisms by which the A_3AR suppresses neutrophil activities was not readily apparent. In this study, we provide evidence that activating the A_3AR signals to inhibit activation of Rac, a small GTPase intimately involved in regulating both neutrophil superoxide production and chemotaxis.

Among the three Rac isoforms (Rac1, 2, and 3), it is generally viewed that Rac2 is the major isoform expressed in neutrophils, and that Rac2 regulates chemoattractant-induced neutrophil functions, including chemotaxis and superoxide production [15,17,18]. However, defects in chemotaxis and superoxide production of neutrophils from Rac2-null mice are further augmented by the additional loss of Rac1 [18]. In addition, Rac1 deficiency alone results in an inability of neutrophils to detect and orient within a chemotactic gradient [19]. Thus, both Rac1 and Rac2 appear to play contributing roles in regulating the pro-inflammatory actions of neutrophils. In the present investigation, Rac activity was not completely inhibited in CP-532,903-pre-treated cells. Since the assay used in our studies did not discriminate between individual Rac isoforms, it remains possible that A_3AR activation resulted in specific inhibition of one of the two Rac isoforms expressed in neutrophils. Such a scenario could explain our previous observations that A_3AR activation with CP-532,903 produces only a 50% inhibition of stimulated superoxide production and only a modest reduction in chemotaxis [5].

We examined whether activation of the A_3AR influences F-actin formation and MAPK activation, two downstream effector system regulated by Rac. In neutrophils and multiple other cell types, Rac regulates actin polymerization involved in cell migration, through effects on Pak1-LIM kinase 1 that inhibits actin depolymerization and gelsolin that regulates actin polymerization [34–36]. Both ERK and p38 signaling are also known to be involved in chemoattractant-induced superoxide generation and migration, although the exact mechanisms remain unclear [37,38]. Using phalloidan staining coupled with fluorescence detection, we found that pre-treating neutrophils with CP-532,903 markedly attenuated fMLP-induced F-actin formation, providing further support for the hypothesis that activating the A_3AR signals to inhibit Rac activation thereby slowing migration. In contrast, we found that CP-532,903 treatment did not alter fMLP-induced activation of ERK or p38 kinases. This observation is consistent with previous findings suggesting that fMLP-induced activation of MAP kinases in neutrophils involves multiple different input signals and is only partially dependent upon Rac activity [15].

Our studies ruled out two potential mechanisms by which stimulating the A_3AR may lead to inhibition of fMLP-stimulated Rac activation and neutrophil activities, including cAMP elevation and alterations in the magnitude and duration of fMLP-induced Ca^{2+} transients. Although traditionally thought to stimulate G_i proteins, it has previously been suggested that the A_3AR increases cAMP production in eosinophils, reportedly through atypical coupling to $G_{s\alpha}$ or through the release of mediators that act upon other G_s protein-coupled receptors [39,40]. Since Ca^{2+} transients were unaffected and MAP kinases continued to be activated, our results further oppose a potential mechanism related to A_3AR-induced cross-desensitization of fMLP receptors, a mechanism that has been suggested to explain the ability of G_i protein-coupled μ and δ opioid receptors to inhibit neutrophil chemotaxis [31,41,42].

The question remains as to the precise mechanism by which stimulating the A_3AR produces selective interference with fMLP-induced Rac activation. The A_3AR has been reported to activate phosphitadylinositol-3-kinase (PI3K) via the release of βγ subunits from $G_{i/o}$ in various cell types leading to activation of ERK 1/2 [43–47]. In our studies, we also observed that activation of the A_3AR produced transient phosphorylation of Erk 1/2 without inducing an increase in the intracellular free Ca^{2+} concentration (Fig. 6), suggesting that the A_3AR might signal to regulate PI3K, phosphoi-

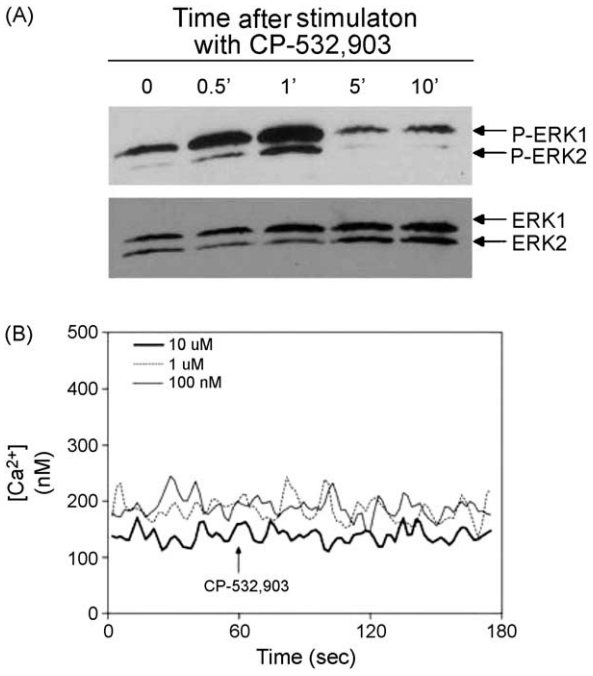

Fig. 6. Treating neutrophils with CP-532,903 (100 nM) directly produced a transient increase in ERK1/2 phosphorylation (A) but did not induce changes in the intracellular $[Ca^{2+}]$ at concentrations as high as 10 μM (B). The data shown are representative of three independent experiments.

nositide metabolism, and Erk1/2 in neutrophils selectively without promoting activation of phospholipase C, Ca^{2+} signaling, and activation of protein kinases C (PKC) isoenzymes. It is well established that activation of neutrophils in response to fMLP and other chemokines involves, in addition to mobilization of intracellular Ca^{2+} and PKC activation, localized generation of phosphoinositides by PI3K at the leading edge, which recruits guanine nucleotide exchange factors (GEFs) that functionally link fMLP receptors to Rac activation. Of particular interest is the major Rac activator in neutrophils P-Rex1 [48–53]. This Rac2-specific GEF, which translocates to the leading edge in areas of Rac2 activation and is synergistically activated by the binding of phosphatidylinositol(3,4,5)triphosphate (PIP_3) and βγ subunits of heterotrimeric G proteins, has been implicated in regulating neutrophil NADPH oxidase activity and chemotaxis in response to a wide variety of extracellular stimuli including fMLP [48–53]. Accordingly, we speculate that pre-activation of the A_3AR in neutrophils may sequester necessary signaling components (PI3K and Rac-specific GEFS such as P-Rex1) away from subsequently stimulated fMLP receptors localized to the leading edge, thereby interfering with Rac activation. Essentially, we predict that activating the A_3AR alters the spatiotemporal signaling events required for fMLP-induced activation of Rac and subsequently Rac-dependent neutrophil functions.

All of our studies in this report were conducted in the presence of adenosine deaminase to remove any effects of endogenous adenosine that might be produced in our assays. This was done in order to isolate the actions of the A_3AR in neutrophils and to parallel our previously published study in which we showed that the A_3AR signals to inhibit neutrophil activation [5]. It is important to note that chemoattractants stimulate the release of ATP and the subsequent production of adenosine that likely influences signaling by various purinergic receptors including the A_3AR [54]. Indeed, it has been proposed by Chen et al. [54] that the release of purines at the leading edge of neutrophils coordinates signaling responses and drives directional migration of the cells.

In conclusion, this study provides evidence that the A_3AR signals in murine neutrophils to inhibit Rac activation in response to the bacterial chemoattractant fMLP. This likely explains the inhibitory effect of A_3AR activation on the pro-inflammatory activities of neutrophils. The precise mechanism by which the A_3AR signals to inhibit Rac activation remains to be identified. However, three possibilities were excluded in this report, namely alterations in Ca^{2+} signaling, cAMP elevation, and receptor cross-desensitization.

Acknowledgements

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References

[1] Nathan C. Points of control in inflammation. Nature 2002;420:846–52.
[2] Nathan C. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol 2006;6:173–82.
[3] Hasko G, Cronstein BN. Adenosine: an endogenous regulator of innate immunity. Trends Immunol 2004;25:33–9.
[4] Hasko G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov 2008;7:759–70.
[5] van der Hoeven D, Wan TC, Auchampach JA. Activation of the A_3 adenosine receptor suppresses superoxide production and chemotaxis of mouse bone marrow neutrophils. Mol Pharmacol 2008;74:685–96.
[6] Ge ZD, Peart JN, Kreckler LM, Wan TC, Jacobson MA, Gross GJ, et al. Cl-IB-MECA [2-chloro-N^6-(3-iodobenzyl)adenosine-5′-N-methylcarboxamide] reduces ischemia/reperfusion injury in mice by activating the A_3 adenosine receptor. J Pharmacol Exp Ther 2006;319:1200–10.
[7] Jordan JE, Thourani VH, Auchampach JA, Robinson JA, Wang NP, Vinten-Johansen J. A_3 adenosine receptor activation attenuates neutrophil function and neutrophil-mediated reperfusion injury. Am J Physiol 1999;277:H1895–905.
[8] Lee HT, Kim M, Joo JD, Gallos G, Chen JF, Emala CW. A_3 adenosine receptor activation decreases mortality and renal and hepatic injury in murine septic peritonitis. Am J Physiol Regul Integr Comp Physiol 2006;291:R959–69.
[9] Montesinos MC, Desai A, Delano D, Chen JF, Fink JS, Jacobson MA, et al. Adenosine A_{2A} or A_3 receptors are required for inhibition of inflammation by methotrexate and its analog MX-68. Arthritis Rheum 2003;48:240–7.
[10] Montesinos MC, Yap JS, Desai A, Posadas I, McCrary CT, Cronstein BN. Reversal of the antiinflammatory effects of methotrexate by the nonselective adenosine receptor antagonists theophylline and caffeine: evidence that the antiinflammatory effects of methotrexate are mediated via multiple adenosine receptors in rat adjuvant arthritis. Arthritis Rheum 2000;43:656–63.
[11] Wan TC, Ge ZD, Tampo A, Mio Y, Bienengraeber MW, Tracey WR, et al. The A_3 adenosine receptor agonist CP-532,903 [N^6-(2,5-dichlorobenzyl)-3′-aminoadenosine-5′-N-methylcarboxamide] protects against myocardial ischemia/reperfusion injury via the sarcolemmal ATP-sensitive potassium channel. J Pharmacol Exp Ther 2008;324:234–43.
[12] Bokoch GM, Zhao T. Regulation of the phagocyte NADPH oxidase by Rac GTPase. Antiox Redox Signal 2006;8:1533–48.
[13] Carstanjen D, Yamauchi A, Koornneef A, Zang H, Filippi MD, Harris C, et al. Rac2 regulates neutrophil chemotaxis, superoxide production, and myeloid colony formation through multiple distinct effector pathways. J Immunol 2005;174:4613–20.
[14] Kim C, Dinauer MC. Rac2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. J Immunol 2001;166:1223–32.
[15] Roberts AW, Kim C, Zhen L, Lowe JB, Kapur R, Petryniak B, et al. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. Immunity 1999;10:183–96.
[16] Ambruso DR, Knall C, Abell AN, Panepinto J, Kurkchubasche A, Thurman G, et al. Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. Proc Natl Acad Sci USA 2000;97:4654–9.
[17] Williams DA, Tao W, Yang F, Kim C, Gu Y, Mansfield P, et al. Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. Blood 2000;96:1646–54.
[18] Gu Y, Filippi MD, Cancelas JA, Siefring JE, Williams EP, Jasti AC, et al. Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. Science 2003;302:445–9.
[19] Sun CX, Downey GP, Zhu F, Koh AL, Thang H, Glogauer M. Rac1 is the small GTPase responsible for regulating the neutrophil chemotaxis compass. Blood 2004;104:3758–65.
[20] Salvatore CA, Tilley SL, Latour AM, Fletcher DS, Koller BH, Jacobson MA. Disruption of the A_3 adenosine receptor gene in mice and its effect on stimulated inflammatory cells. J Biol Chem 2000;275:4429–34.
[21] Lieber JG, Webb S, Suratt BT, Young SK, Johnson GL, Keller GM, et al. The in vitro production and characterization of neutrophils from embryonic stem cells. Blood 2004;103:852–9.
[22] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[23] Geijsen N, van Delft S, Raaijmakers JA, Lammers JW, Collard JG, Koenderman L, et al. Regulation of p21rac activation in human neutrophils. Blood 1999;94:1121–30.
[24] Li S, Yamauchi A, Marchal CC, Molitoris JK, Quilliam LA, Dinauer MC. Chemoattractant-stimulated Rac activation in wild-type and Rac2-deficient murine neutrophils: preferential activation of Rac2 and Rac2 gene dosage effect on neutrophil functions. J Immunol 2002;169:5043–51.
[25] Bokoch GM. Chemoattractant signaling and leukocyte activation. Blood 1995;86:1649–60.
[26] Tintinger G, Steel HC, Anderson R. Taming the neutrophil: calcium clearance and influx mechanisms as novel targets for pharmacological control. Clin Exp Immunol 2005;141:191–200.
[27] Price LS, Langeslag M, ten Klooster JP, Hordijk PL, Jalink K, Collard JG. Calcium signaling regulates translocation and activation of Rac. J Biol Chem 2003;278:39413–21.
[28] Anderson R, Visser SS, Ramafi G, Theron AJ. Accelerated resequestration of cytosolic calcium and suppression of the pro-inflammatory activities of human neutrophils by CGS 21680 in vitro. Br J Pharmacol 2000;130:717–24.
[29] Ali H, Richardson RM, Haribabu B, Snyderman R. Chemoattractant receptor cross-desensitization. J Biol Chem 1999;274:6027–30.
[30] Didsbury JR, Uhing RJ, Tomhave E, Gerard C, Gerard N, Snyderman R. Receptor class desensitization of leukocyte chemoattractant receptors. Proc Natl Acad Sci USA 1991;88:11564–8.
[31] Grimm MC, Ben-Baruch A, Taub DD, Howard OM, Resau JH, Wang JM, et al. Opiates transdeactivate chemokine receptors: δ and μ opiate receptor-mediated heterologous desensitization. J Exp Med 1998;188:317–25.

[32] Tomhave ED, Richardson RM, Didsbury JR, Menard L, Snyderman R, Ali H. Cross-desensitization of receptors for peptide chemoattractants. Characterization of a new form of leukocyte regulation. J Immunol 1994;153:3267–75.
[33] Zhang N, Yang D, Dong H, Chen Q, Dimitrova DI, Rogers TJ, et al. Adenosine A_{2A} receptors induce heterologous desensitization of chemokine receptors. Blood 2006;108:38–44.
[34] Arcaro A. The small GTP-binding protein Rac promotes the dissociation of gelsolin from actin filaments in neutrophils. J Biol Chem 1998;273:805–13.
[35] Azuma T, Witke W, Stossel TP, Hartwig JH, Kwiatkowski DJ. Gelsolin is a downstream effector of Rac for fibroblast motility. EMBO J 1998;17:1362–70.
[36] Edwards DC, Sanders LC, Bokoch GM, Gill GN. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat Cell Biol 1999;1:253–9.
[37] Dewas C, Fay M, Gougerot-Pocidalo MA, El-Benna J. The mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway is involved in formyl-methionyl-leucyl-phenylalanine-induced p47phox phosphorylation in human neutrophils. J Immunol 2000;165:5238–44.
[38] Zu YL, Qi J, Gilchrist A, Fernandez GA, Vazquez-Abad D, Kreutzer DL, et al. p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-α or FMLP stimulation. J Immunol 1998;160:1982–9.
[39] Knight D, Zheng X, Rocchini C, Jacobson M, Bai T, Walker B. Adenosine A_3 receptor stimulation inhibits migration of human eosinophils. J Leuk Biol 1997;62:465–8.
[40] Walker BA, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D, et al. Adenosine A_3 receptor expression and function in eosinophils. Am J Respir Cell Mol Biol 1997;16:531–7.
[41] Rogers TJ, Steele AD, Howard OM, Oppenheim JJ. Bidirectional heterologous desensitization of opioid and chemokine receptors. Ann NY Acad Sci 2000;917:19–28.
[42] Zhang N, Hodge D, Rogers TJ, Oppenheim JJ. Ca^{2+}-independent protein kinase Cσ mediate heterologous desensitization of leukocyte chemokine receptors by opioid receptors. J Biol Chem 2003;278:12729–36.
[43] Hammarberg C, Fredholm BB, Schulte G. Adenosine A_3 receptor-mediated regulation of p38 and extracellular-regulated kinase ERK1/2 via phosphatidylinositol-3′-kinase. Biochem Pharmacol 2004;67:129–34.
[44] Hammarberg C, Schulte G, Fredholm BB. Evidence for functional adenosine A_3 receptors in microglia cells. J Neurochem 2003;86:1051–4.
[45] Schulte G, Fredholm BB. Human adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. Mol Pharmacol 2000;58:477–82.
[46] Schulte G, Fredholm BB. Signaling pathway from the human adenosine A_3 receptor expressed in Chinese hamster ovary cells to the extracellular signal-regulated kinase 1/2. Mol Pharmacol 2002;62:1137–46.
[47] Schulte G, Fredholm BB. Signalling from adenosine receptors to mitogen-activated protein kinases. Cell Signal 2003;15:813–27.
[48] Barber MA, Donald S, Thelen S, Anderson KE, Thelen M, Welch HC. Membrane translocation of P-Rex1 is mediated by G protein βγ subunits and phosphoinositide 3-kinase. J Biol Chem 2007;282:29967–76.
[49] Dong X, Mo Z, Bokoch G, Guo C, Li Z, Wu D. P-Rex1 is a primary Rac2 guanine nucleotide exchange factor in mouse neutrophils. Curr Biol 2005;15:1874–9.
[50] Mayeenuddin LH, Garrison JC. Phosphorylation of P-Rex1 by the cyclic AMP-dependent protein kinase inhibits the phosphatidylinositiol (3,4,5)-trisphosphate and G βγ-mediated regulation of its activity. J Biol Chem 2006;281:1921–8.
[51] Welch HC, Coadwell WJ, Ellson CD, Ferguson GJ, Andrews SR, Erdjument-Bromage H, et al. P-Rex1, a PtdIns(3,4,5)P3- and G βγ regulated guanine-nucleotide exchange factor for Rac. Cell 2002;108:809–21.
[52] Welch HC, Condliffe AM, Milne LJ, Ferguson GJ, Hill K, Webb LM, et al. P-Rex1 regulates neutrophil function. Curr Biol 2005;15:1867–73.
[53] Zhao T, Nalbant P, Hoshino M, Dong X, Wu D, Bokoch GM. Signaling requirements for translocation of P-Rex1, a key Rac2 exchange factor involved in chemoattractant-stimulated human neutrophil function. J Leuk Biol 2007;81:1127–36.
[54] Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, et al. ATP release guides neutrophil chemotaxis via $P2_{Y2}$ and A_3 receptors. Science 2006;314:1792–5.