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Quercetin Reduces Neutrophil Recruitment Induced by CXCL8, LTB_4, and fMLP: Inhibition of Actin Polymerization

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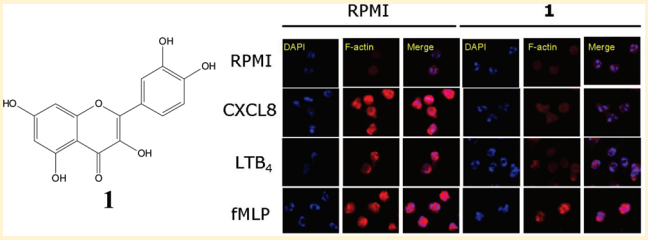
ABSTRACT: Recent in vitro data have suggested that the flavonoid quercetin (**1**) does not affect the functioning of neutrophils. Therefore, we evaluated in vivo and in vitro whether or not **1** affects neutrophil function, focusing on recruitment. The in vivo treatment with **1** inhibited in a dose-dependent manner the recruitment of neutrophils to the peritoneal cavity of mice induced by known chemotatic factors such as CXCL1, CXCL5, LTB_4, and fMLP. Furthermore, **1** also inhibited in a concentration-dependent manner the chemoattraction of human neutrophils induced by CXCL8, LTB_4, and fMLP in a Boyden chamber. In vitro treatment with **1** did not affect human neutrophil surface expression of CXCR1, CXCR2, BLT1, or FLPR1, but rather reduced actin polymerization. These results suggest that **1** inhibits actin polymerization, hence, explaining the inhibition of neutrophil recruitment in vivo and in vitro and highlighting its possible usefulness to diminish excessive neutrophil migration during inflammation.



Quercetin (**1**) is known as a prototype antioxidant flavonoid, and most of its widely recognized biological effects are related to antioxidant properties by scavenging oxygen radicals, inhibiting lipid peroxidation as well as protein and DNA oxidation, and chelating metal ions.[1,2]

Although not completely understood, it is likely that there is a link between the antioxidant and anti-inflammatory effects of **1**.[3] In this sense, **1** also exhibits analgesic effects in models of inflammatory overt pain-like behavior and hyperalgesia,[3,4] inhibits inflammatory edema,[3] and inhibits inflammation-induced production of cytokines,[3] prostaglandin E_2,[5] inducible nitric oxide synthase expression, and NFκB activation.[5] Thus, the published data support the anti-inflammatory effects of **1**.

An important component of the inflammatory response is phagocytosis and the elimination of infectious agents by recruited neutrophils.[6] There are divergent data on the effect of **1** in neutrophils. For instance, quercetin inhibited myeloperoxidase activity in vitro[7] and in a model of UVB-induced skin inflammation in hairless mice,[8] but had no effect in the carrageenin paw inflammation-induced myeloperoxidase increase.[3] Myeloperoxidase is an important enzyme for neutrophil microbicidal activity and is also used as a marker of neutrophil recruitment.[9] Furthermore, recent in vitro data have indicated that treatment with **1** did not alter the lipopolysaccharide-induced cell surface expression of the adhesion molecules L-selectin (CD62L) and β2 integrin (CD11b/Mac 1),[10] which are related to the rolling and firm adhesion of neutrophils, respectively.[6] Therefore, it seems unlikely that **1** would inhibit neutrophil recruitment.[10] On the other hand, **1** inhibits fMLP-induced increase of intracellular calcium,[10] which is necessary for actin polymerization and consequently neutrophil migration.[6] However, these earlier studies did not investigate whether or not **1** inhibits neutrophil recruitment.[10]

In view of the information presented above, it was investigated as to whether quercetin inhibits neutrophil recruitment in vivo and in vitro and if such activity depends on diminishing the

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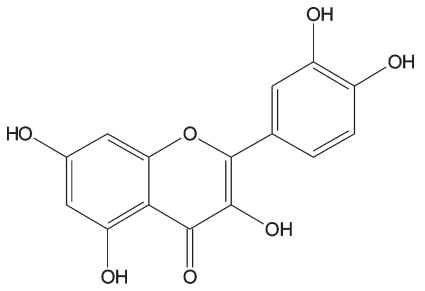
expression of receptors for chemotatic inflammatory mediators and/or actin polymerization.



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RESULTS AND DISCUSSION

Quercetin (1) Inhibits Neutrophil Recruitment in Vivo Induced by Different Stimuli. In the first series of experiments, whether or not **1** inhibits neutrophil recruitment in vivo was addressed. Mice were treated with **1** (30−300 mg/kg, subcutaneous [sc], diluted in saline with 20% Tween 80) 30 min before intraperitoneal (ip) injection of saline (200 μL, ip), CXCL1 (3 ng/mouse, ip, Figure 1A), CXCL5 (30 ng/mouse, ip, Figure 1B), LTB_4 (25 ng/mouse, ip, Figure 1C), or fMLP (formyl-methionyl-leucyl-phenylalanine, 3 μg/mouse, ip, Figure 1D). The stimuli were chosen to represent different classes of mediators; CXCL1 and CXCL5 were used because they are chemotatic cytokines, LTB_4 is a lipid mediator, and fMLP is a peptide produced by bacteria and causes inflammation. All these mediators have established roles in inflammation.[11−14] Quercetin doses of 100 and 300 mg/kg inhibited neutrophil recruitment induced by all four stimuli, with a tendency of a greater effect by the 300 mg/kg dose (Figure 1). Inhibition by both doses was significant compared to the lower dose of 30 mg/kg of **1** except as in Figure 1D with fMLP stimulus, in which there were no significant differences between the doses used. There was a tendency of reduction of neutrophil recruitment but no significant effect with 30 mg/kg of **1** in Figure 1A and B with CXCL1 and CXCL5 stimulus, respectively. These results demonstrate that **1** has significant activity in diminishing neutrophil recruitment in vivo independently of the inflammatory stimuli applied, suggesting wide applicability. The divergent data on indirect neutrophil recruitment determination using myeloperoxidase activity could be related to differences in the experimental approach such as in vitro versus in vivo,[3,7] and UVB radiation versus carrageenin stimulus.[3,8] In the carrageenin paw inflammation model, we did not detect any effect of **1** on myeloperoxidase activity.[3] Nevertheless, the dose of carrageenin was chosen on the basis of mechanical hyperalgesia evaluation rather than neutrophil recruitment (with usual doses of 100 μg versus 500 μg, respectively),[3,11] **1** was administered intraperitoneally[3] not subcutaneously (present study), and neutrophil recruitment was evaluated in the paw skin[3] instead of peritoneal cavity (present study). All these variables might influence the results and together increase the differences encountered among them.

Treatment with Quercetin (1) Inhibits Human Neutrophil Chemoattraction in the Boyden Chamber. Neutrophil migration to the inflammatory focus is a multifactorial event depending on several steps such as rolling and adhesion of neutrophils to endothelial cells followed by transmigration. Another important step in neutrophil recruitment is chemotaxis induced by a group of inflammatory mediators well known as chemoattractants. Thus, it was assessed next whether **1** is able to inhibit the direct chemoattraction of human neutrophils in vitro. The chemokines CXCL1 and CXCL5 of mice correspond to CXCL8 in humans, and LTB_4 and fMLP are active in mice and humans; their main receptors are BLT1 and FLPR1, respectively.[12−15] Human neutrophils express CXCR1 (CXCL8 receptor), CXCR2 (CXCL8 receptor), BLT1, and FLPR1.[16−18] Therefore, CXCL8, LTB_4, and fMLP are able to chemoattract neutrophils in vitro. The incubation of human neutrophils with **1** (30 min of pretreatment at concentrations of 10, 30, and 100 nM, and diluted in RPMI medium with 2% DMSO) inhibited in a concentration-dependent manner the chemoattraction induced by CXCL8 (10 ng/mL, Figure 2A), LTB_4 (10^{-7} M, Figure 2B), and fMLP (10^{-7} M, Figure 2C). Concentrations of 30 and 100 nM of **1** significantly inhibited the neutrophil chemoattraction induced by all stimuli (Figure 2). The inhibition observed with concentrations of 30 (Figure 2C) and 100 nM (Figure 2A and C) of **1** were statistically significant compared to its lowest concentration (10 nM). The concentration of 10 nM of **1** inhibited the chemoattraction induced by fMLP (Figure 2C) without affecting the chemoattraction induced by CXCL8 (Figure 2A) or LTB_4 (Figure 2B). Higher concentrations of **1** (300 and 1000 nM) were also tested in the chemotaxis assay. However, these did not show an increase in inhibition of chemotaxis as compared to 100 nM of **1** (data not shown). Therefore, quercetin inhibits the direct chemoattraction of human neutrophils in the Boyden chamber.

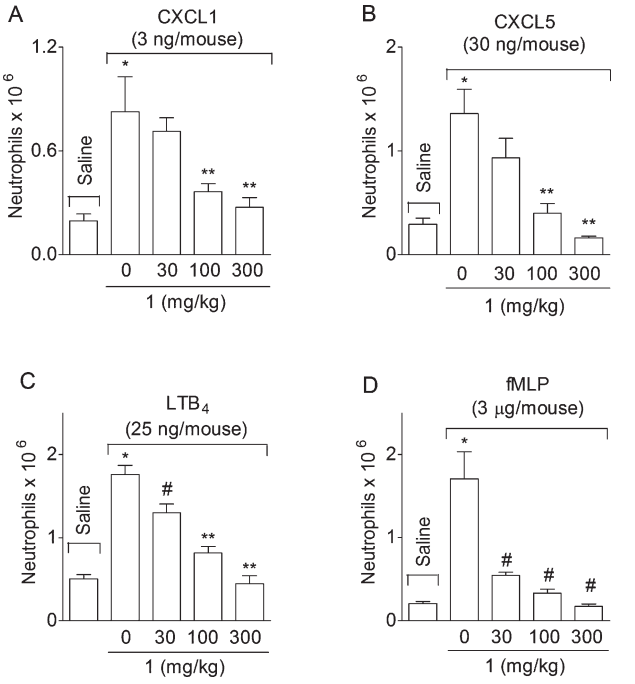

Figure 1. Quercetin (**1**) inhibits neutrophil recruitment in vivo induced by different stimuli. Panels A−D: Mice were treated with **1** (30−300 mg/kg, sc, 30 min) or vehicle (indicated as 0; 20% Tween 80 in saline) before intraperitoneal injection of saline (200 μL), CXCL1 (3 ng/mouse, panel A), CXCL5 (30 ng/mouse, panel B), LTB_4 (25 ng/mouse, panel C), or fMLP (3 μg/mouse, panel D). Neutrophil recruitment was determined 6 h after stimulus injection ($n = 6$). [$^{*}p < 0.05$ compared with the saline group, $^{\#}p < 0.05$ compared to the vehicle group, and $^{**}p < 0.05$ compared to the vehicle group and the dose of 30 mg/kg of **1** (one-way ANOVA followed by Tukey's t test)].

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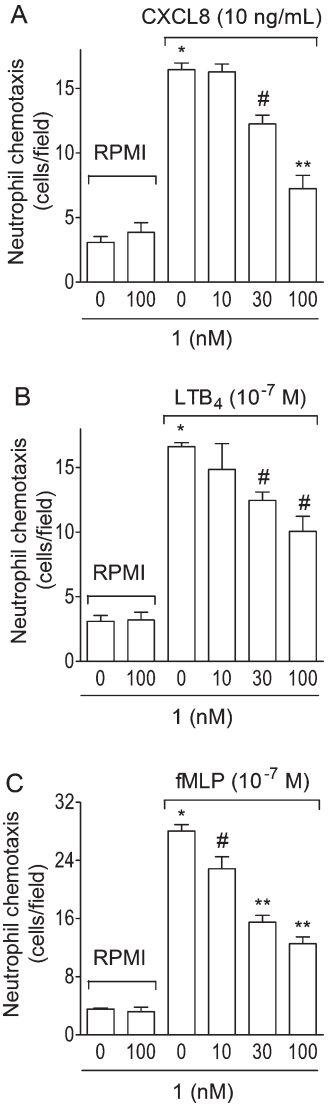

Figure 2. Treatment with quercetin (**1**) inhibits human neutrophil chemoattraction in the Boyden chamber. Panels A−C: Human neutrophils were incubated with **1** (10−100 nM, 30 min) or vehicle (indicated as 0; RPMI with 2% DMSO) before stimulating with CXCL8 (10 ng/mL, panel A), LTB_4 (10^{-7} M, panel B), or fMLP (10^{-7} M, panel C). Neutrophil chemotaxis was determined 2 h after stimulus in slices stained using a commercial kit consisting of a variation of Romanowsky stain (n = 4 wells per group, 5 random fields per well). [$^{*}p < 0.05$ compared with the saline group, $^{\#}p < 0.05$ compared to the vehicle group, and $^{**}p < 0.05$ compared to the concentration of 10 nM of **1** (one-way ANOVA followed by Tukey's t test)].

Quercetin (1) Did Not Alter the Human Neutrophil Surface Expression of Receptors for Chemotatic Mediators. Neutrophils are chemoattracted by CXCL8, LTB_4, and fMLP through their respective receptors expressed in the neutrophil membrane.[12,13] Therefore, it was assessed if **1** inhibits the surface expression of CXCR1, CXCR2, BLT1, or FLPR1. A concentration of 100 nM of **1** was chosen on the basis of the results shown in Figure 2. Despite inhibiting the chemoattraction of human neutrophils induced by CXCL8, LTB_4, and fMLP, **1** did not alter the expression of their receptors: CXCR1 (Figure 3A), CXCR2 (Figure 3B), BLT1 (Figure 3C), and FLPR1 (Figure 3D). Although treatment with **1** inhibited neutrophil recruitment in vivo (Figure 1; in mice) and in vitro (Figure 2; human neutrophils), these inhibitory effects may not be explained by diminishing the expression of chemotatic receptors in the surface of human neutrophils (Figure 3).

Quercetin (1) Reduced Human Neutrophil Actin Polymerization Induced by CXCL8, LTB_4, and fMLP. Human neutrophils underwent the same treatment protocol described above followed by actin polymerization evaluation. Actin polymerization was induced by CXCL8, LTB_4, and fMLP, as shown in the representative fluorescence images (Figure 4A) and analysis (Figure 4B). Nuclei were stained using DAPI. The actin polymerization induced by CXCL8, LTB_4, and fMLP was inhibited by the preincubation of neutrophil with **1**, as shown in the representative fluorescence images (Figure 4A) and analysis (Figure 4B).

Actin polymerization is a crucial event for neutrophil cytoskeleton reorganization during recruitment. This activation of the cytoskeleton depends on the increase of cytoplasmatic concentrations of calcium, which, at least for fMLP, were demonstrated to be inhibited by **1**.[10]

In the present study, it was demonstrated that quercetin inhibits neutrophil recruitment in vivo and in vitro and is supportive of treatment with **1** affecting neutrophil function, despite previous data suggesting the opposite.[10] The mechanisms inhibited by **1** are not related to inhibition of receptor surface expression, but rather are probably related to modulation of the internal signaling cascades activated by agonist binding to receptors, of which the consequence is actin polymerization and neutrophil movement toward an inflammatory focus. It is noteworthy that activation of phosphatidylinositol 3-kinase (PI_3K) has been described as an important signaling event that increases cytoplasmic calcium inducing actin polymerization and, therefore, cellular migration.[15] This PI_3K-mediated pathway is important in CXCL8 and LTB_4 chemoattraction of neutrophils.[17,18] Recent evidence has demonstrated that **1** inhibits PI_3K signaling,[19−21] which, together with the present results, suggests that PI_3K could be a target of quercetin to inhibit neutrophil migration. Others have demonstrated that **1** inhibits PI_3K-dependent cancer cell proliferation,[19] tumor necrosis factor-alpha-induced up-regulation of matrix metalloproteinase-9,[20] arsenite-induced cyclooxygenase-2 expression by rat liver epithelial cells,[21] and apoptosis evasion in human salivary adenoid cystic carcinoma.[22] In the case of fMLP, inhibition of PI_3K delays neutrophil chemoattraction and actin polymerization, but does not reduce the final number of recruited neutrophils. On the other hand, inhibition of the mitogen-activated protein (MAP) kinase p38 blocks fMLP-induced neutrophil chemotaxis.[23] Other studies have detected that **1** inhibits p38-dependent events such as platelet spreading of collagen and fibrinogen[24] and osteoblast chemotaxis.[25] Thus, it is possible that quercetin diminishes neutrophil recruitment by inhibiting signaling kinases such as PI_3K[15,19−21] and p38[24,25] involved in neutrophil cytoskeleton reorganization,[15,23] which are activated by chemoattractants including CXCL8, LTB_4, and fMLP, respectively.[12−18,23]

Therefore, it is likely that quercetin (**1**) inhibits neutrophil recruitment by inhibiting cellular signaling responsible for actin polymerization, indicating that treatment with **1** is a conceivable approach to control excessive neutrophil recruitment during inflammation and to prevent neutrophil-mediated tissue lesions.

EXPERIMENTAL SECTION

Chemicals. The following materials were obtained from the sources indicated: Quercetin (**1**) at 95% purity from Acros (Pittsburgh, PA);

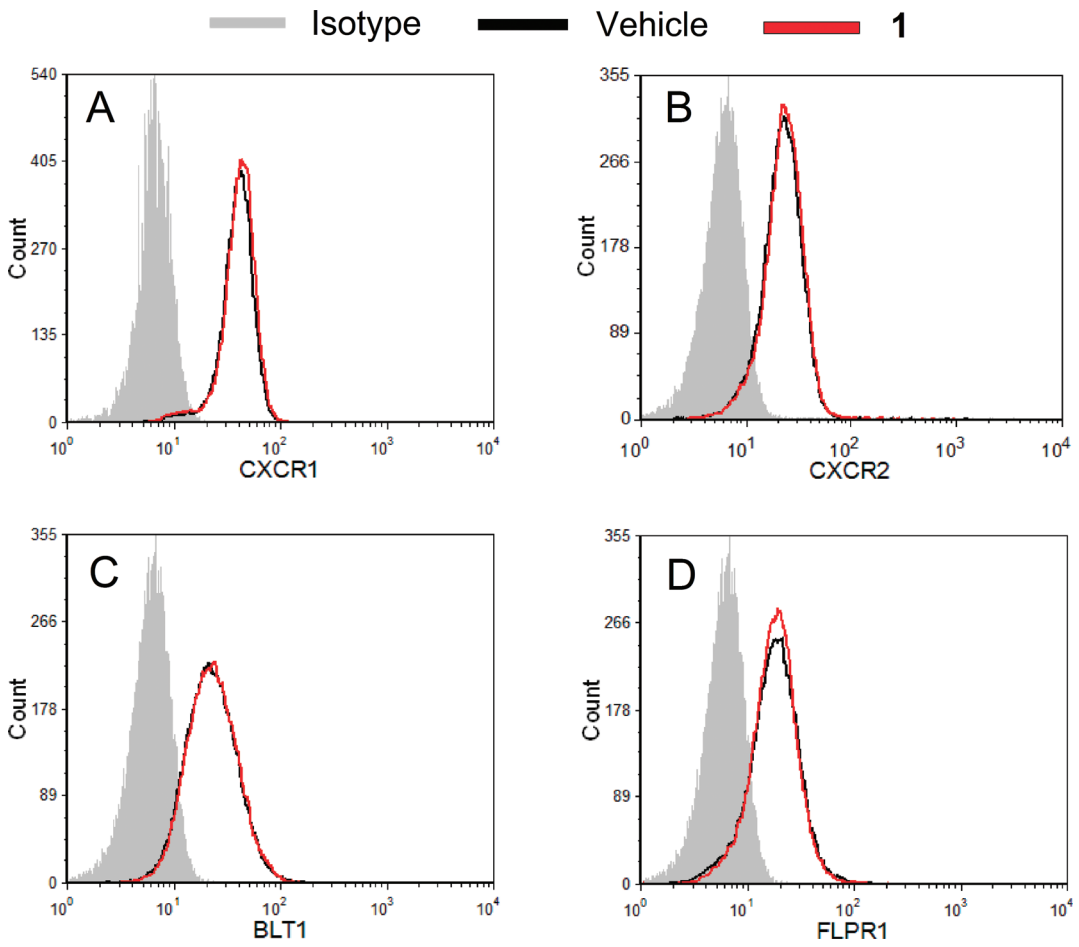

Figure 3. Quercetin (**1**) did not alter the human neutrophil surface expression of receptors for chemotatic mediators. Panels A−D: Human neutrophils were incubated with **1** (100 nM, 30 min) or vehicle (RPMI with 2% DMSO) for 30 min and then incubated with isotype IgG control antibody, or anti-CXCR1 (A), anti-CXCR2 (B), anti-BLT1 (C), or anti-FLPR1 (D) antibodies, followed by flow cytometry analysis (n = 3).

recombinant murine CXCL1 and CXCL5 and recombinant human CXCL8 from PeproTech Inc. (Rocky Hill, NJ); LTB_4, anti-CXCR1, anti-CXCR2, anti-BLT1, anti-FLPR1, or control antibodies from R&D Systems (Minneapolis, MN); fMLP, Hanks−Hanks balanced salt solution (HBSS), EDTA, Percoll, and NH_4Cl from Sigma (St. Louis, MO); a commercial kit consisting of a variation of Romanowsky stain from Dade Behring (Deerfield, IL); methanol from Merck (Darmstadt, Germany); rhodamine-phalloidin from Molecular Probes (Eugene, OR).

Animals. Male Swiss mice (25−30 g) from the University of Sao Paulo, Ribeirao Preto, Sao Paulo, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12:12 h and were kept at 21 °C. Animal care and handling procedures were approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences of Ribeirao Preto (University of Sao Paulo; protocol no. 04.1.951.53.1). All efforts were made to minimize the number of animals used and their suffering.

In Vivo Neutrophil Recruitment. Neutrophil migration was assessed 6 h after CXCL1 (3 ng/mouse), CXCL5 (30 ng/mouse), LTB_4 (25 ng/mouse), or fMLP (3 μg/mouse) intraperitoneal stimulus. The animals were killed, and the cells present in the peritoneal cavity were harvested by introducing 2.0 mL of phosphate-buffered saline (PBS) containing 1 mM of EDTA. Total counts were performed with a Newbauer chamber, and differential cell counts were carried out on cytocentrifuge slides (Cytospin 3; Shandon Southern Products, Astmoore, UK) stained using a commercial kit consisting of a variation of Romanowsky stain. The results were expressed as the number of neutrophils.[26]

Neutrophil Isolation and Chemotaxis. Human neutrophils were isolated by four-layer Percoll gradient from human peripheral blood and with approval of the Human Ethics Committee of the Faculty of Medicine of Ribeirao Preto (University of Sao Paulo; protocol no. 12664/2006).[13] The purity of neutrophil preparation was >98% determined by Rosenfeld-colored Cytospin. The chemotaxis assay was performed using a 48-well microchamber (Neuro Probe, Boyden chamber). The stimuli and negative control were added to the lower chambers. A 5 μm pore polycarbonate membrane (Neuro Probe) was placed between the upper and lower chambers, and 5×10^4 neutrophils previously treated for 30 min with **1** (10−100 nM) were added to the top chambers. Cells were allowed to migrate into the membrane for 1 h at 37 °C, 5% CO_2. Following incubation, the membrane was washed in PBS, fixed in methanol 80%, and stained using a commercial system consisting of a variant of Romanowsky stain (Dade Behring). Each well-associated membrane area was scored using light microscopy to count the intact cells present in five random fields. The results are expressed as the number of neutrophils per field.[13]

Flow Cytometry Analysis. Purified human neutrophils from peripheral blood were treated with **1** (100 nM) for 30 min and then incubated with peridinin-chlorophyll-protein-conjugated (PerCP) anti-CXCR1, phycoerythrin-conjugated (PE) anti-CXCR2, PE anti-BLT1, PE anti-FLPR1, or IgG isotype control antibodies (R&D Systems). The cells were washed, fixed, and analyzed by flow cytometry using a BD Biosciences FACSort flow cytometer.[27]

F-Actin Assembly (actin polymerization). Purified human neutrophils from peripheral blood were treated with **1** (100 nM) for

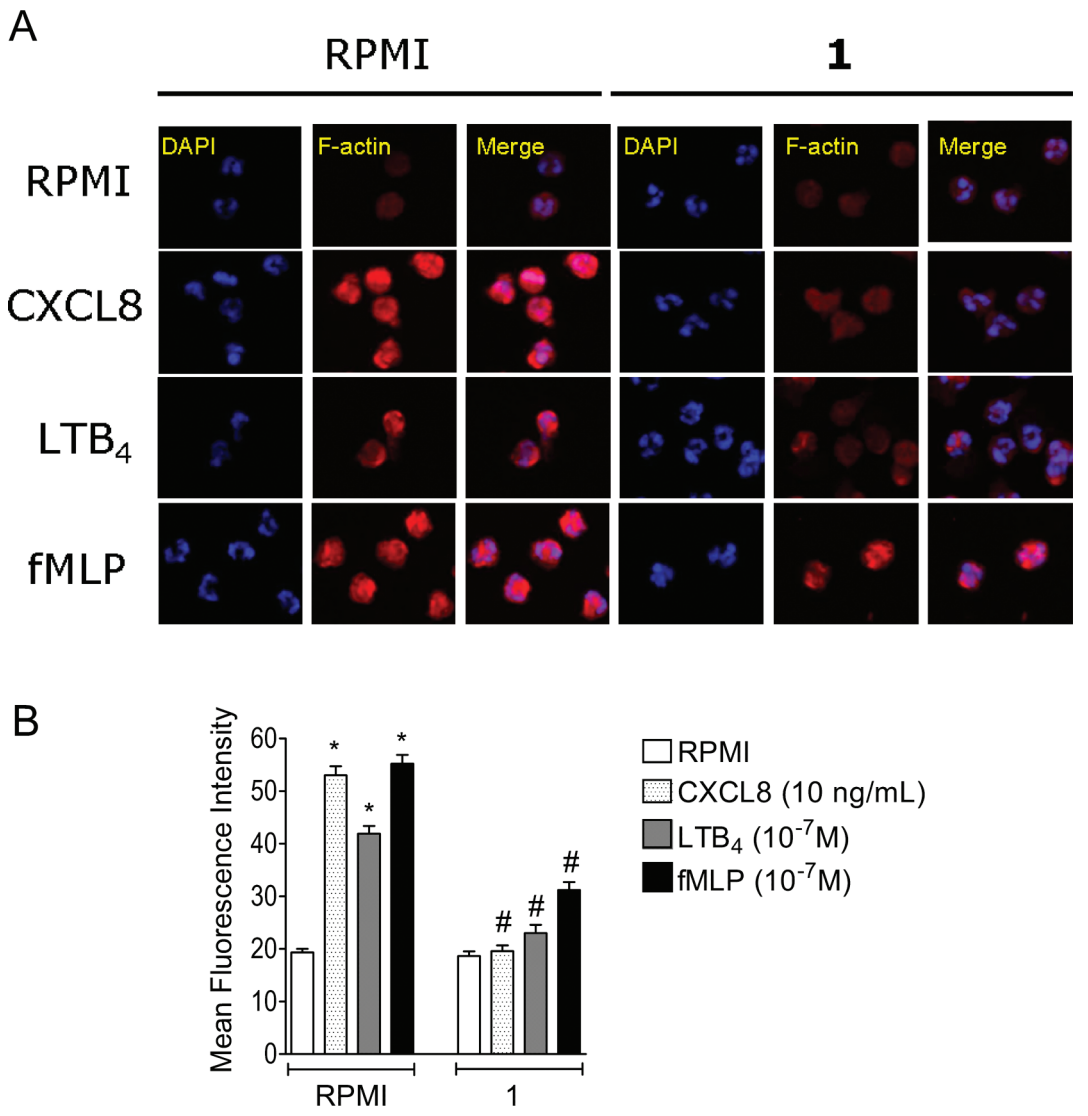

Figure 4. Quercetin (**1**) reduced human neutrophil F-actin polymerization induced by CXCL8, LTB_4, and fMLP. Panels A and B: Human neutrophils were incubated with **1** (100 nM, 30 min) or vehicle (RPMI with 2% DMSO) before stimulating with CXCL8 (10 ng/mL), LTB_4 (10^{-7} M), or fMLP (10^{-7} M). F-Actin polymerization was determined by immunofluorescence (n = 3 slides, 5 random fields each). [$^{*}p < 0.05$ compared with the saline group, and $^{\#}p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey's t test)].

30 min, followed by incubation with CXCL8 (10 ng/mL), LTB_4 (10^{-7} M), or fMLP (10^{-7} M) at 37 °C for 5 min. Afterward, the reaction was stopped with 2% paraformaldehyde followed by fixation on glass slides by Cytospin centrifugation (Cytospin 3; Shandon Southern Products). After centrifugation, cells were again fixed during 20 min with 4% paraformaldehyde, followed by permeabilization and staining with rhodamine-phalloidin (Molecular Probes). Microscopic analysis of fluorescent images was performed using an Olympus BX40-F4 epifluorescence microscope. The mean fluorescence intensity (MFI) was determined from a linear measurement of the fluorescence of individual cells. All cells of at least five randomly chosen fields of each slide were analyzed, three slides per group.[27]

Experimental Protocols. Mice received subcutaneous (30, 100, and 300 mg/kg) treatment with quercetin (**1**) or vehicle (20% Tween 80 in saline) 30 min before inflammatory stimulus. The doses of inflammatory stimuli were previously determined in our laboratory in pilot studies and based on previous work.[13,14,26−29] Neutrophil recruitment was evaluated 6 h after ip injection of CXCL1 (3 ng/mouse), CXCL5 (30 ng/mouse), LTB_4 (25 ng/mouse), or fMLP (3 μg/mouse). Human neutrophils were incubated with **1** (10, 30, and 100 nM) or vehicle (RPMI with 2% DMSO) 30 min and then assayed for chemotaxis in a Boyden chamber and for receptor cell surface expression by flow cytometry or for actin polymerization/F-actin assembly by immunefluorescence using CXCL8 (10 ng/mL), LTB_4 (10^{-7} M), or fMLP (10^{-7} M) as stimuli.

Statistical Analysis. Results are presented as means ± SEM of experiments made on six animals per group (Figure 1), five random fields of each well, four wells per group (Figure 2), in triplicate (Figure 3), or five random fields of each slide, three slides per group (Figure 4) and are representative of two separate experiments. Differences between groups were evaluated by analyses of variance (one-way ANOVA) followed by the Tukey test. Statistical differences were considered to be significant at $p < 0.05$.

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