A Biased Non-G\(\alpha_i\) OXE-R Antagonist Demonstrates That G \(\alpha_i\) Protein Subunit Is Not Directly Involved in Neutrophil, Eosinophil, and Monocyte Activation by 5-Oxo-ETE

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A Biased Non-Gαi OXE-R Antagonist Demonstrates That Gαi Protein Subunit Is Not Directly Involved in Neutrophil, Eosinophil, and Monocyte Activation by 5-Oxo-ETE

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G protein–coupled chemotaxtractant receptors, such as the 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-oxo-ETE) receptor (OXE-R), are able to switch on Gαβγ protein-dependent and β-arrestin–related signaling traits. However, which of these signaling pathways are truly important for the chemotaxtractant functions in leukocytes is not clarified yet. As we recently reported, Gue1654 is a unique Gβγ-biased OXE-R antagonist having no inhibitory activity on Gαi-related signaling, which makes Gue1654 an unprecedented tool for assessing the involvement of G protein subunits in chemotaxtractant receptor function. β-arrestin2 recruitment was studied in OXE-R–overexpressing HEK293 cells using bioluminescence resonance energy transfer assays. Activation of leukocytes was assessed by flow cytometric assays and by immunofluorescence microscopy. Leukocyte capture to endothelial cells was addressed under physiological flow conditions. We found that Gue1654 blocks β-arrestin2 recruitment in HEK293 cells overexpressing OXE-R and ERK1/2 phosphorylation in human eosinophils and neutrophils. Furthermore, Gue1654 was able to prevent several 5-oxo-ETE–triggered functional events in eosinophils and neutrophils, such as activation of CD11b/CD18 integrins, oxidative burst, actin polymerization, and interaction with endothelial cells. In addition, Gue1654 completely prevented 5-oxo-ETE–induced Ca2+ flux and chemotaxis of human primary monocytes. All of these leukocyte responses to 5-oxo-ETE, except ERK1/2 phosphorylation and oxidative burst, were likewise prevented by pertussis toxin. Therefore, we conclude that chemotaxtractant receptors require Gαi subunits only as adaptors to transactivate the Gβγ heteromers, which then act responsible for cell activation. Finally, our data characterize Gue1654 as a non-Gαi-biased antagonist of OXE-R that provides a new basis for therapeutic intervention in inflammatory diseases that involve activation of eosinophils, neutrophils, and monocytes. The Journal of Immunology, 2014, 192: 000–000.

One very powerful chemotaxtractant of leukocytes is 5-oxo-eicosatetraenoic acid (5-oxo-ETE; for review, see Ref. 4), and the 5-oxo-ETE receptor (OXE-R) is likewise a PTX-sensitive Gαi-coupled GPCR (5, 6). 5-Oxo-ETE, belonging to the eicosanoids, is an oxygenated 5-lipoxygenase metabolite of arachidonic acid and is released by monocytes, dendritic cells, and platelets, and by endothelial, epithelial, and airway smooth muscle cells (7). 5-Oxo-ETE robustly activates eosinophils, neutrophils, monocytes, and basophils (8–10); in particular, it induces Ca2+ mobilization, actin polymerization, CD11b expression, and L-selectin shedding of eosinophils (8). Additionally, 5-oxo-ETE drives infiltration of eosinophils and neutrophils into human skin after intradermal injection (11). Although further elucidation of the pathophysiologic role of 5-oxo-ETE is hampered by the fact that no rodent ortholog of OXE-R has been detected to date, it may be assumed that 5-oxo-ETE plays a pivotal role in inflammatory diseases such as asthma, allergic rhinitis, arthritis, and psoriasis (7, 12); accordingly, specific OXE-R antagonists such as recently reported by Gore et al. (13) are likely to be beneficial in treating these diseases.

We have recently reported on Gue1654, a newly identified biased OXE-R antagonist that selectively disrupts Gβγ, but does not affect Gαi signaling triggered by 5-oxo-ETE (14). In the current study, we elucidated the general importance of Gαi protein activation in the function of human eosinophils, neutrophils, and monocytes by exploiting this to date unique non-Gαi–biased OXE-R antagonist. Furthermore, we explored the selectivity of Gue1654 regarding G protein–independent OXE-R signaling pathways.
such as those initiated by β-arrestin recruitment and ERK1/2 phosphorylation.

Materials and Methods
Chemicals and Abs
Unless specified, all laboratory reagents were purchased from Sigma-Aldrich (Vienna, Austria). 5-Oxo-ETE was purchased from Cayman (Ann Arbor, MI). Human eotaxin/CCL11 and IL-8 were from PeproTech (London, U.K.). Mouse mAb 24 (mAb 24, 24-activation-sensitive CD1 integrin Ab) was from Hygult Biotechnology (Uden, The Netherlands). Goat anti-mouse and anti-rabbit IgG secondary Abs conjugated with Alexa Fluor 488 and phalloidin–Texas Red were supplied by Invitrogen (Vienna, Austria). Activation-sensitive mouse anti-CD11b (FITC) was from BioLegend (Fell, Germany); anti-CD16 (PE), CellFix, and FACs-Flow were obtained from BD Biosciences (Vienna, Austria). Vectashield/DAPI mounting medium was from Vector Laboratories (Burlingame, CA). The Ab against Thr<sup>202</sup>/Tyr<sup>204</sup> phospho-p44/42 MAPKs (ERK1/2) was from Cell Signaling Technology (Boston, MA). Ultra V blocking solution was purchased from Lab Vision (Fremont, CA), and Ab diluted from Dako (Carpinteria, CA). Assay buffer was made from Dulbecco’s modified PBS (with 0.9 mM/L Ca<sup>2+</sup> and 0.5 mM/L Mg<sup>2+</sup>; Invitrogen), 0.1% BSA, 10 mM/L HEPES, and 10 mM/L glucose (pH 7.4). Fixative solution was prepared by adding 9 ml distilled water and 30 ml FACs-Flow to 1 ml CellFix. Drugs were dissolved in ethanol, distilled water, or DMSO and further diluted in assay buffer to produce a final concentration of the solvents of <0.1%

Preparation of human peripheral blood leukocytes
Blood was taken after written informed consent from healthy nonatopic volunteers who were not taking any medication, according to a protocol approved by the Institutional Review Board of the Medical University of Graz. Preparation of polymorphonuclear leukocytes (PMNL: neutrophils and eosinophils) and PBMC (monocytes, dendritic cells, basophils, and lymphocytes) were prepared by dextran sedimentation of erythrocytes and centrifugation over Histopaque density gradients, as described before (10, 15). Eosinophils and monocytes were further purified by respective Ab cocktails coupled to magnetic beads (StemCell Technologies, Vancouver, Canada). Resulting purities and viabilities were >98% (10).

Culture of endothelial cells
Human lung microvascular endothelial cells were purchased from Lonza (Verviers, Belgium) and were maintained in EGM-2 MV Bullet medium (Lonza) with 5% FCS. All culture surfaces were precoated with 1% gelatin for 1 h at 37˚C to promote endothelial cell attachment and growth. The medium was changed every second day, and cells were passaged upon reaching 90% confluence (5–6 d). The cells were used between 5 and 10 passages (16).

Culture and transfection of HEK293-β-arrestin2-GFP<sup>2</sup> cells
HEK293 cells stably expressing GFP<sup>2</sup>-labeled β-arrestin2 (βARR2) were provided by J. Mosolff Mathiesen (University of Copenhagen). Cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml gentamicin (G418). For bioluminescence resonance energy transfer (BRET) assay, cells were transiently transfected with pCDNA3.1+-OXE-R-RLuc using the gene pulser Xcell. Cells were transiently cultivated in DMEM supplemented with 10% FCS, 100 U/ml penicillin, provided by J. Mosolff Mathiesen (University of Copenhagen). Cells were transfected with pCMV-βGFP<sup>2</sup> cells that were transiently coexpressing OXE-R-RLuc, as described before (14).

Activation of CD11b and CD18 integrin subunits
Activation of CD11b and CD18 subunits in leukocytes was determined by flow cytometry using FITC-labeled CBRM1/5 Ab and the unconjugated mAb 24, which recognize only the activated conformation of the respective integrin subunits. Purified neutrophils and eosinophils were treated as indicated at 37˚C for 30 min in the presence of CBRM1/5-FITC (1:40) or mAb 24 (1:50) or isotype control Ab. Activation of CD18 subunit was detected by subsequent incubation with Alexa Fluor 488-labeled secondary Ab (4 μg/ml).

Respiratory burst
PMNL (5 × 10<sup>5</sup> cells/ml) were stimulated by 5-oxo-ETE at indicated concentrations in the presence of 2'-7’-dichlorodihydrofluorescein diacetate (final concentration 10 μM) for 10 min at 37˚C, followed by immediate fixation with ice-cold 2.5% CellFix. Respiratory burst of leukocytes was quantified by oxidation of nonflourescent 2’-7’-dichlorodihydrofluorescein to fluorescent 2’-7’-dichlorofluorescein in flow cytometry (18).

F-actin staining of adherent leukocytes
Purified neutrophils and eosinophils were treated as indicated and were allowed to adhere to chamber slides precoated with 5 μg/ml fibronectin (15). Adherent cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100, and nonspecific binding sites were blocked with 1% BSA/PBS. Cells were incubated with phallolidin–Texas Red (5 U/ml) for 30 min at room temperature. The slides were then mounted with Vectashield mounting medium. Image quantification was performed in observer-blinded fashion using ImageJ software (National Institutes of Health, Bethesda, MD).

Leukocyte adhesion to endothelial cells under flow conditions
Human pulmonary microvascular endothelial cells (4 × 10<sup>5</sup>/substrate) were grown into confluent monolayers on VenaEC bioships (Cellix, Dublin, Ireland). Endothelial monolayers were superfused with suspensions of 3 × 10<sup>6</sup>/ml purified neutrophils or eosinophils at 0.5 dyn/cm<sup>2</sup> for 3 min at 37˚C C in a OKOLAB H201-T1 heated cage. Cells were treated as indicated. Leukocyte capture to endothelial cells was monitored by phase contrast 3.5–5 min later on a Zeiss Axiovert 40 CFL microscope and Zeiss A-Plan 10×/0.25 Ph1 lens, using a Hamamatsu ORCA-03G digital camera and CellixVenaFlux software. Cell adhesion was quantified by computerized image analysis using DucoCell analysis software (Cellix) by a person unaware of the treatment conditions (15, 16).

Monocyte Ca<sup>2+</sup> flux
PBMC (10<sup>5</sup> cell/ml) were loaded with 2 μM fluo-3-acetoxyethyl ester Ca<sup>2+</sup>-sensitive dye in the presence of 0.02% pluronic F-127 for 60 min at room temperature. Cells were then labeled with anti-CD14 PerCP Ab. Monocytes were identified as CD14-positive cells, and changes in intracellular Ca<sup>2+</sup> levels were detected by flow cytometry as the increase of fluorescence of fluo-3 in the FL1-channel, as previously described (10).

Monocyte migration
Migration of freshly isolated human monocytes (2 × 10<sup>5</sup> cells per insert) in RPMI 1640 medium (containing 5 mM HEPES, 1% nonessential amino acids, and 1% sodium pyruvate) was assessed using Transwell inserts with 5-μm pore size (Corning) for 18 h at 37˚C. After removal of nontransmigrated cells using a cotton swab, migrated cells attached to the bottom side of the filter were fixed with 3.7% paraformaldehyde for 30 min at 4˚C. Filters were mounted on microscope slides with DAPI-containing mounting medium, as previously described (19). Migration was analyzed by Zeiss Axiovert 40 CFL microscope and Zeiss A-Plan 10×/0.25 Ph1 lens; five images were acquired per filter using a Hamamatsu ORCA-03G digital camera. Migration was quantified by counting DAPI-labeled nuclei by a person unaware of the respective treatment using ImageJ software (National Institutes of Health).

Statistical analyses
Data are shown as mean ± SEM for n observations. Experiments with human primary leukocytes were repeated four to eight times with cells from different donors. Statistical analysis was performed with GraphPad Prism software using one-way ANOVA for repeated measurements, followed by Tukey’s multiple comparison test or two-way ANOVA for repeated measurements, followed by Bonferroni’s post hoc test. The p values <0.05 were considered as statistically significant.
Results

Gue1654 prevents OXE-R βARR2 recruitment and ERK1/2 phosphorylation

βARR2 is known to serve as an intracellular adaptor protein mediating G protein–independent signaling of GPCRs, and, in fact, OXE-R has been described to induce βARR2 recruitment (20). We investigated the impact of Gue1654 on the βARR2 recruitment of OXE-R in BRET assays. To this end, we used HEK293-βARR2-GFP² cells that transiently coexpressed the OXE-R fused to RLuc. Incubation of cells with 5-oxo-ETE in concentrations ranging from 100 pM to 100 μM for 10 min induced BRET with a pEC₅₀ value of 7.22 ± 0.1. To determine whether βARR2 recruitment to the OXE-R is dependent on Gαi activation, HEK293 cells were pretreated with PTX (50 ng/ml) or its vehicle for 18 h. PTX did not attenuate βARR2 recruitment to OXE-R, as 5-oxo-ETE still displayed a pEC₅₀ of 7.14 ± 0.11, similar to that of non-PTX–treated HEK293 cells (Fig. 1A). In contrast, Gue1654 pretreatment (3–30 μM) for 30 min resulted in concentration-dependent reduction of the 5-oxo-ETE–induced BRET signal by decreasing the maximal response (Fig. 1B).

Interestingly, the antagonistic effect of Gue1654 was not attenuated, but even accentuated by PTX as compared with vehicle-treated cells (Fig. 1B, 1C). For instance, the maximal response to 5-oxo-ETE at 30 μM antagonist was 54.6 ± 1.5% of the control response in the absence of PTX, but only 30.4 ± 3.5% after PTX treatment (p < 0.05, n = 4). These results demonstrated that Gue1654 is an antagonist of OXE-R that also inhibits Gαᵢ-dependent βARR2 recruitment.

β-arrestins have been recently described as functional scaffold proteins interacting with GPCRs in a G protein–independent fashion, by linking GPCRs to MAPK such as ERK1/2, thereby triggering important intracellular signaling pathways (21, 22). In eosinophil activation, ERK1/2 was reported to be a key player among other kinases such as p38 MAPK and protein kinase C (23). Both in neutrophils and eosinophils, 5-oxo-ETE (10–300 nM) induced the phosphorylation of ERK1/2 within 2 min as profoundly as PMA (500 nM), which was applied as positive control. At 1 μM Gue1654 slightly reduced and at higher concentrations almost abolished the effect of 5-oxo-ETE (Fig. 2A, 2B). Next, we tested for the Gαi dependence of the 5-oxo-ETE–induced ERK1/2 phosphorylation. We found that PTX pretreatment (3 μg/ml for 1 h) did not modify the 5-oxo-ETE–induced ERK1/2 phosphorylation in neutrophils; however, in eosinophils, PTX partially—although not significantly—inhibited ERK1/2 phosphorylation in response to 5-oxo-ETE (Fig. 2C, 2D). These data suggested that Gue1654 prevents OXE-R βARR2 recruitment and ERK1/2 phosphorylation in a Gαᵢ-independent manner.

Gue1654, the biased OXE-R antagonist, blocks 5-oxo-ETE–induced neutrophil and eosinophil activation

The 5-oxo-ETE–stimulated ERK1/2 phosphorylation was found to be crucial for the activation of eosinophils resulting in, for example, CD11b upregulation and chemotaxis (23). Chemokine-stimulated activation of integrin subunits such as CD11b and CD18 is a crucial step in leukocyte recruitment into the inflamed tissue (24). Thus, we investigated the inhibitory effect of Gue1654 on this mechanistic level of leukocyte activation in specific flow cytometric assays for both integrin subunits. As control stimuli, we used IL-8 for neutrophils and eotaxin for eosinophils. Activation of CD11b subunits in neutrophils, and likewise in eosinophils, was highly induced by 5-oxo-ETE and was almost completely prevented by 10 and 30 μM Gue1654 (Fig. 3). The CD18 subunit in neutrophils was only modestly activated by 5-oxo-ETE, which was reversed by Gue1654 treatment (Fig. 3A). In eosinophils, 5-oxo-ETE greatly enhanced the activation of CD18 subunits, which was abolished by 10 and 30 μM Gue1654 (Fig. 3B).

Next, we examined the impact of Gue1654 on a further effector function of leukocytes, respiratory burst. 5-Oxo-ETE stimulated respiratory burst of neutrophils and eosinophils concentration dependently. Gue1654 at 1 and 10 μM concentration had no effect on the neutrophil respiratory burst, but partially reversed that of eosinophils; at 30 μM Gue1654 abolished the 5-oxo-ETE–triggered respiratory burst in both cell populations (Fig. 4A). PTX pretreatment (3 μg/ml) for 1 h only partially decreased respiratory burst in both leukocytes (Fig. 4B). We probed the involvement of the Gαᵢ-dependent phospholipase C (PLC) pathway by incubating the cells with a respective inhibitor (1 μM) for 20 min; the active compound U73122 completely prevented the production of reactive oxygen species in neutrophils and eosinophils, whereas its inactive analog U73343 showed no inhibition (Fig. 4C). These findings suggest that, in addition to PTX-sensitive Gαᵢ/Gβγ heterotrimer, OXE-R activation stimulates respiratory burst through alternative pathways, possibly involving Gαq family members.
Neutrophil and eosinophil adhesion is blocked by Gue1654

The generation of adhesive cellular structures containing polymerized actin filaments and clusters of activated integrins is a crucial step in the process of leukocyte adhesion and crawling (24). 5-Oxo-ETE as a potent chemoattractant of eosinophils and neutrophils has been shown to induce actin polymerization of eosinophils by flow cytometric quantitation of F-actin (8). Vehicle-treated neutrophils already showed a few smaller filopodia that was further triggered by 5-oxo-ETE stimulation (Fig. 5). In neutrophils preincubated with Gue1654, we saw a concentration-dependent reduction in filopodia (Fig. 5A). Purified eosinophils were round without showing cellular protrusions after vehicle treatment; however, 5-oxo-ETE induced very intense actin polymerization and strong polarization of the cell shape with appearance of several filopodia. Gue1654 reduced actin polymerization and markedly prevented formation of cellular protrusions induced by 5-oxo-ETE (Fig. 5). Gue1654, when applied alone, did not modulate adhesive structure formation in leukocytes. 5-Oxo-ETE has not yet been shown to stimulate leukocyte–endothelial interaction. In this study, we treated neutrophils and purified eosinophils with 5-oxo-ETE and perfused them through channels containing confluent layers of human pulmonary microvascular endothelial cells. Under control conditions, a mean of 10 neutrophils and 15 eosinophils per high-power field was captured to the endothelial cell layer, as observed by live-cell imaging (Fig. 6). 5-Oxo-ETE at 300 nM caused a 2.5-fold increase in adhesion of both cell types (Fig. 6A, 6B). This effect was com-

![Figure 2](image1)

**FIGURE 2.** 5-Oxo-ETE–induced phosphorylation of ERK1/2 in neutrophils and eosinophils is prevented by Gue1654 but is only slightly decreased by PTX. Polymorphonuclear leukocyte preparations were preincubated with the OXE-R antagonist Gue1654 for 10 min (A, B) or with PTX for 1 h (C, D), followed by stimulation with indicated concentrations of 5-oxo-ETE or PMA for 2 min. Phosphorylation of ERK1/2 was determined by indirect flow cytometric staining. Eosinophils were distinguished from neutrophils as CD16-negative cells. Responses are expressed as mean fluorescence intensity and are shown as mean + SEM; n = 5. *p < 0.05 versus vehicle.

![Figure 3](image2)

**FIGURE 3.** 5-Oxo-ETE–induced CD11b/CD18 activation is reversed by Gue1654. Polymorphonuclear leukocyte preparations were preincubated with Gue1654 for 10 min, followed by stimulation with indicated concentrations of 5-oxo-ETE for 30 min. Activation of CD11b and CD18 subunits on neutrophils (A) and eosinophils (B) was measured with flow cytometry. IL-8 and eotaxin were used as specific control stimuli, respectively. Eosinophils were distinguished from neutrophils as CD16-negative cells. Responses were expressed as percentage of baseline fluorescence. Data are shown as mean + SEM, n = 4. *p < 0.05 versus vehicle.
parable to that of eotaxin (3 nM) with respect to eosinophil adhesion, whereas neutrophil adhesion to endothelial cells was more pronounced by 5-oxo-ETE than IL-8 (3 nM) treatment, as we observed in previous studies (15, 16). Preincubation of leukocytes with Gue1654 caused a concentration-dependent decrease in adhesion to the endothelial layer (Fig. 6A, 6B), and at 30 μM Gue1654 completely prevented the capture of neutrophils and eosinophils triggered by 5-oxo-ETE (Fig. 6C). Importantly, when applied alone at this concentration, Gue1654 did not affect the basal adhesion of leukocytes. When investigating the dependence

![FIGURE 4. Gue1654 prevents the 5-oxo-ETE–induced respiratory burst. Production of reactive oxygen species in neutrophils and purified eosinophils was determined by flow cytometry using 2'-7'-dichlorodihydrofluorescein diacetate labeling. Cells were pre-treated with indicated concentrations of Gue1654 for 10 min (A), pertussis toxin for 1 h (B), or the active (U73122) and inactive (U73343) form of the PLC inhibitor for 10 min (C), followed by stimulation with indicated concentrations of 5-oxo-ETE for 10 min. Responses were expressed as percentage of baseline fluorescence. Data are shown as mean + SEM, n = 4. *p < 0.05 versus vehicle.](http://www.jimmunol.org/)

![FIGURE 5. Gue1654 prevents the formation of adhesive structures by neutrophils and eosinophils. Leukocytes were preincubated with 30 μM Gue1654 for 10 min, followed by treatment with 300 nM 5-oxo-ETE for 12 min in chamber slides precoated with 5 mg/ml fibronectin. Polymerization of F-actin was detected with phalloidin–Texas Red staining, and nuclei were stained with DAPI. (A) Filopodia formation per cell was quantified by ImageJ software from the micrographs. Data are shown as mean + SEM, and stainings were repeated four to five times. **p < 0.001 versus vehicle, *p < 0.05, **p < 0.01 versus 300 nM 5-oxo-ETE. (B) Representative micrographs are shown of neutrophil and eosinophil adhesive structure formation.](http://www.jimmunol.org/)
on Gαiβγ heterotrimer of leukocyte adhesion to endothelial cells, we found that pretreatment of the leukocytes with 3 μg/ml PTX for 1 h almost completely blocked the effect of 5-oxo-ETE (Fig. 6D, 6E), suggesting that this complex leukocyte response is greatly dependent on Gαiβγ subunits released upon Gαiβγ heterotrimer activation.

Gue1654 inhibits monocyte Ca2+ mobilization and completely prevents migration

Monocytes are important effector cells in several inflammatory diseases and have also been found to respond to 5-oxo-ETE in assays of intracellular Ca2+ mobilization, shape change, and migration (9, 25). Monocytes express OXE-R to a similar extent as neutrophils, as we have previously shown at the mRNA level (10). Therefore, we investigated Gue1654 for antagonistic activity of OXE-R in human peripheral blood monocytes. We confirmed the 5-oxo-ETE–stimulated intracellular Ca2+ mobilization (Fig. 7A), and found that a 10-min pretreatment with 10 μM Gue1654, just as 3 μg/ml PTX for 1 h, completely inhibited the 5-oxo-ETE–induced Ca2+ flux (Fig. 7B). This finding reflects our previous observations that Gue1654 was able to abolish the 5-oxo-ETE–induced Ca2+ mobilization in eosinophils and neutrophils (14).

To address the inhibitory potential of Gue1654 in monocyte migration, purified monocytes were allowed to migrate toward 5-oxo-ETE (10–300 nM) for 18 h in Transwell plates, followed by enumerating the fluorescence-labeled nuclei of transmigrated monocytes (Fig. 8). We observed a concentration-dependent increase of monocyte mobility; notably, 5-oxo-ETE proved to be as effective a chemoattractant for monocytes as MCP-1 at 3 nM concentration (Fig. 8A). Following preincubation of monocytes with Gue1654 for 10 min, we found a reduced migration already at 300 nM antagonist, followed by a total

![FIGURE 6. Gue1654 attenuates the capture of neutrophils and eosinophils to human lung microvascular endothelial cells under physiological flow conditions. Neutrophils (A) and purified eosinophils (B) were preincubated with indicated concentrations of Gue1654 or vehicle for 10 min and then treated with 300 nM 5-oxo-ETE for 5 min. Leukocytes were then superfused over confluent pulmonary microvascular endothelial cell layers. Bars show the mean + SEM, n = 3. *p < 0.05, **p < 0.01 versus 300 nM 5-oxo-ETE. (C) Representative images taken 3 min after the start of the perfusion are shown for neutrophils and eosinophils. Neutrophils (D) and purified eosinophils (E) were preincubated with 3 μg/ml PTX or vehicle for 1 h and then treated with 300 nM 5-oxo-ETE for 5 min. Data are mean + SEM, n = 3–4. **p < 0.01 versus 300 nM 5-oxo-ETE.](http://www.jimmunol.org/)

![FIGURE 7. Gue1654 prevents the 5-oxo-ETE–dependent intracellular Ca2+ mobilization in human monocytes. (A) Mononuclear cells were stimulated with vehicle, the indicated concentrations of 5-oxo-ETE, or 1 nM MCP-1. Changes in intracellular Ca2+ levels in CD14-positive monocytes were detected by flow cytometry and are shown as mean + SEM percentage of baseline, n = 4. *p < 0.05, **p < 0.01 versus vehicle. (B) Mononuclear cells were preincubated with indicated concentrations of Gue1654 for 10 min or with PTX for 1 h, followed by stimulation with 300 nM 5-oxo-ETE. Data are expressed as mean percentage of baseline Ca2+ level + SEM, n = 4. **p < 0.01 versus vehicle; *p < 0.05, ***p < 0.01 versus 300 nM 5-oxo-ETE.](http://www.jimmunol.org/)
block of 5-oxo-ETE–induced monocyte migration with increasing concentrations of Gue1654 (Fig. 8B). Selected images of transmigrated monocytes with vehicle, 5-oxo-ETE, and 3 μM Gue1654 plus 5-oxo-ETE treatments are shown in Fig. 8C. These data show that, like in granulocytes, Gue1654 acts as a full antagonist of OXE-R–mediated Ca2+ mobilization and migration of monocytes. Additionally, PTX preincubation also blocked 5-oxo-ETE–triggered monocyte migration (Fig. 8D). These observations collectively suggest that Gbg but not Gai subunits control chemotactic movement of these cells (Fig. 9).

Discussion
Gue1654 is a small-molecule antagonist of OXE-R that uniquely uncouples Gαi- and Gbg-mediated downstream signaling events in human neutrophils and eosinophils, and in recombinant cells overexpressing OXE-R, as we have recently described (14). Gue1654 antagonizes OXE-R in a mode that preserves Gαi-dependent inhibition of adenylyl cyclase but completely impairs the Gbg heterodimer-triggered Ca2+ mobilization, inositol monophosphate release, and gating of G protein–regulated inwardly rectifying K+ channels (14). Our present findings extend the antagonistic profile of Gue1654 and reveal that—besides inhibition of Gbg signaling traits—Gue1654 precludes the G-protein–independent bARR2 recruitment upon 5-oxo-ETE stimulation. Consequently, the whole ensemble of OXE-R–triggered signaling events, except that of activated Gαi protein subunits, is inhibited by Gue1654. Intriguingly, this mode of biased antagonism is apparently sufficient to prevent the 5-oxo-ETE–induced functional activation of human leukocytes.

Chemoattractant receptors are usually GPCRs coupled to Gαi proteins. However, the only known direct effect of Gαi activation

FIGURE 8. Gue1654 blunts 5-oxo-ETE–induced migration of monocytes. (A) Purified monocytes were placed into Transwell plate inserts with 5-μm pore size and were allowed to migrate toward vehicle, indicated concentrations of 5-oxo-ETE, or 3 nM MCP-1 in the bottom wells for 18 h. Transmigration was evaluated by labeling the nuclei of monocytes with DAPI and microscopic analysis. Data are shown as percentage of the migratory response toward vehicle, mean + SEM, n = 4–6. *p < 0.05, **p < 0.01 versus vehicle. (B) Monocytes were preincubated with indicated concentrations of Gue1654 for 10 min, followed by stimulation with 100 nM 5-oxo-ETE. Data are expressed as mean + SEM, n = 6–8. **p < 0.01 versus vehicle; #p < 0.05, ##p < 0.01 versus 100 nM 5-oxo-ETE. (C) Representative fluorescence micrographs are shown. (D) Monocytes were pretreated with 3 μg/ml PTX for 1 h, followed by stimulation with 100 nM 5-oxo-ETE. Data are expressed as mean + SEM, n = 7. ***p < 0.001 versus vehicle, ##p < 0.01 versus 100 nM 5-oxo-ETE.

FIGURE 9. Schematic summary of OXE-R–mediated leukocyte responses and proposed involvement of G protein subtypes and bARR2. 5-Oxo-ETE reduces cAMP accumulation in a PTX-sensitive, but Gue1654-insensitive manner, suggesting a direct role of Gαi in OXE-R–mediated adenylate cyclase inhibition (data in Ref. 14). Most leukocyte responses, including Ca2+ flux, actin polymerization, integrin activation, adhesion, and migration, are blocked by both PTX and Gue1654, demonstrating that these responses are mediated by Gbg heteromers. ERK1/2 activation is sensitive to Gue1654, but is not prevented by PTX, which might be compatible with a role of bARR2. 5-Oxo-ETE–induced oxidative burst is abolished after PLC inhibition, but it is only partially sensitive to PTX and is less potently affected by Gue1654 than other leukocyte responses. Gαq and Gα15/16 are known to activate PLC and thus might be involved in OXE-R–mediated oxidative burst.
is inhibition of adenylyl cyclase and subsequent decrease of intracellular cAMP level. Initial observations suggest that Goi activation on its own is not sufficient to induce chemotaxis; instead, the subsequent liberation of Gi/Gbg heterodimer is responsible for initiating directional migration of cells (2, 3). Conversely, blockade of Gi/Gbg subunit signaling by using the small-molecule inhibitor galene was reported to prevent neutrophil chemotaxis triggered by IL-8 and IMLP (26). Apart from G proteins, 7-transmembrane receptors also use β-arrestin–dependent signaling pathways that are currently being uncovered (27). In this study, we found that 5-oxo-ETE potently induced βARK2 recruitment, and this was attenuated by Gue1654. Whether OXE-R signaling also involves β-arrestin1, as seen with class-B GPCRs, or selectively relies on βARK2 (class A receptors) remains to be elucidated. The new concept of β-arrestin signaling merges the classical desensitization/internalization functions of these proteins with more recently described aspects of β-arrestins as multifunctional adaptor proteins; β-arrestins have been reported also to mediate signals via MAPK, NF-k, and phosphoinositide 3-kinase, which can additionally contribute to the functional outcome of receptor activation (22, 27). In fact, we found that 5-oxo-ETE stimulated ERK1/2 phosphorylation in neutrophils and eosinophils in a PTX-insensitive manner, whereas Gue1654 effectively prevented ERK1/2 phosphorylation, which might be compatible with a role of βARK2 in this response. In contrast, 5-oxo-ETE phosphorylation of p40 MAPK had earlier been reported to be PTX sensitive (28). The importance of β-arrestins and associated multikinase signaling complex is well established in mediating chemotaxis of different cell types involving human macrophages, microglia, and vascular smooth muscle cells (29–31). This might not be the case for 5-oxo-ETE stimulation of granulocyte adhesion to endothelial cells and monocyte migration, as PTX abolished both of these cellular functions in the current study. Similar findings were previously reported for F-actin formation and phosphoinositide 3-kinase activation (32).

ERK1/2 phosphorylation is necessary for the upregulation of CD11b/CD18 integrins in eosinophils after chemoattractant stimulation (23). We observed in this study that 5-oxo-ETE effectively induced activation of CD11b and CD18 integrin subunits in eosinophils and neutrophils, which was entirely prevented by Gue1654. MAPKs also play a crucial role in the mediation of oxidative burst. Interestingly, we found that PTX only partially inhibited respiratory burst in granulocytes as induced by 5-oxo-ETE, which coincided with a lower inhibitory potency of Gue1654 as compared with CD11b/18 activation (Fig. 3) or adhesion (Fig. 6A). This was further underpinned by the observation that the 5-oxo-ETE–induced respiratory burst was abolished by the PLC inhibitor U73122. Therefore, we speculate that 5-oxo-ETE respiratory burst in neutrophils and eosinophils was abolished by the PLC inhibitor U73122. Therefore, we speculate that 5-oxo-ETE respiratory burst in neutrophils and eosinophils as induced by 5-oxo-ETE, which coincided with the classical desensitization/internalization functions of these proteins with more recently described aspects of β-arrestins as multifunctional adaptor proteins; β-arrestins have been reported also to mediate signals via MAPK, NF-k, and phosphoinositide 3-kinase, which can additionally contribute to the functional outcome of receptor activation (22, 27).

In conclusion, our results show that the small-molecule antagonist Gue1654 is an effective blocker of 5-oxo-ETE–induced and OXE-R–mediated activation of human neutrophils, eosinophils, and monocytes, including biologically substantial cellular responses such as formation of reactive oxygen species, adhesion to endothelium, and chemotaxis. The efficacy of Gue1654 as an antagonist of these responses is remarkable in light of its known bias toward Gi/Gbg protein–mediated signaling, and as we have also shown in this work, the active Gi/Gbg subunit does not play a direct role in these leukocyte responses and that its function is restricted to liberation of the Gi/Gbg heteromer upon nucleotide exchange. Gue1654 does not affect the PTX-sensitive Gi/Gbg-specific signaling traits, for example, inhibition of cAMP formation, but still robustly prevents major functional outcomes of 5-oxo-ETE–induced activation of eosinophils, neutrophils, and monocytes. Therefore, our data clearly demonstrate that most 5-oxo-ETE–triggered leukocyte responses rely on Gi/Gbg signaling without an involvement of βARK2 (Fig. 9).

To our knowledge, Gue1654 is the first-ever published small-molecule antagonist of OXE-R (14). Despite its limited potency with EC50 values of 1–3 μM, depending on the leukocyte response examined, Gue1654 is a highly promising pharmacophore that might serve as a starting point for further optimization and development of highly potent and selective OXE-R antagonists that might be suitable for clinical evaluation in humans.

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