

Immunopathology and Infectious Diseases

Resolvin D1 Receptor Stereoselectivity and Regulation of Inflammation and Proresolving MicroRNAs

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Resolution of acute inflammation is an active process that involves the biosynthesis of specialized proresolving lipid mediators. Among them, resolvin D1 (RvD1) actions are mediated by two G protein-coupled receptors (GPCRs), ALX/FPR2 and GPR32, that also regulate specific microRNAs (miRNAs) and their target genes in novel resolution circuits. We report the ligand selectivity of RvD1 activation of ALX/FPR2 and GPR32. In addition to RvD1, its aspirin-triggered epimer and RvD1 analogs each dose dependently and effectively activated ALX/FPR2 and GPR32 in GPCR-overexpressing β -arrestin systems using luminescence and electric cell-substrate impedance sensing. To corroborate these findings *in vivo*, neutrophil infiltration in self-limited peritonitis was reduced in human ALX/FPR2-overexpressing transgenic mice that was further limited to 50% by RvD1 treatment with as little as 10 ng of RvD1 per mouse. Analysis of miRNA expression revealed that RvD1 administration significantly up-regulated miR-208a and miR-219 in exudates isolated from ALX/FPR2 transgenic mice compared with littermates. Overexpression of miR-208a in human macrophages up-regulated IL-10. In comparison, in ALX/FPR2 knockout mice, RvD1 neither significantly reduced leukocyte infiltration in zymosan-induced peritonitis nor regulated miR-208a and IL-10 in these mice. Together, these results demonstrate the selectivity of RvD1 interactions with receptors ALX/FPR2 and GPR32. Moreover, they establish a new molecular circuit that is operative in the resolution of acute inflammation activated by the proresolving mediator RvD1 involving specific GPCRs and miRNAs. (*Am J Pathol* 2012, 180:2018–2027; DOI: 10.1016/j.ajpath.2012.01.028)

Chemical mediators regulate acute inflammation and resolution, exerting their actions via cell surface G protein-coupled receptors (GPCRs).^{1,2,3} Results from this laboratory demonstrated that resolution of self-limited inflammation is an active process that involves local and temporal biosynthesis of a new genus of specialized proresolving lipid mediators. Specialized proresolving lipid mediators include several structurally distinct families, including lipoxins from arachidonic acid, resolvins of the E-series, resolvins of the D-series, protectins, and, most recently, maresins, which are enzymatically biosynthesized from the ω -3 fatty acids eicosapentaenoic acid and docosahexaenoic acid (DHA) (for a recent review, see the article by Serhan³). Specialized proresolving lipid mediators are bioactive chemical autacoids, carry potent and protective actions in disease models, and act on specific cellular targets in a stereoselective manner.

Specialized proresolving lipid mediators exert their actions via cell surface GPCRs.³ Lipoxin A₄ (LXA₄) interacts with ALX/FPR2,^{4–6} signals to stop further polymorphonuclear neutrophil (PMN) infiltration, and stimulates nonphlogistic monocyte recruitment⁷ and macrophage phagocytosis of apoptotic PMNs.^{8,9} In humans, the aspirin-triggered 15-epimer of LXA₄ and the expression level of ALX/FPR2 in skin blisters delineated between resolving versus delayed resolution.¹⁰ A member of the D-series resolvins, namely,

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resolvin D1 (RvD1), biosynthesized from the n-3 essential fatty acid DHA, was first identified in resolving inflammatory exudates from acute self-limited murine peritonitis.¹¹ RvD1 biosynthesis and structure were elucidated,¹¹ and its complete stereochemistry was established as 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid.¹²

Low-dose aspirin treatment triggers epimers in lipoxins and resolvins, initiating novel aspirin-triggered biosynthetic pathways. For example, these aspirin-triggered routes lead to the biosynthesis of 15R-LXA₄¹³ and 17R-RvD1¹¹ and to the inhibition of classic cyclooxygenase pathway products, eg, prostaglandins and thromboxanes.^{3,14} These aspirin-triggered endogenous epimers of natural anti-inflammatory and proresolving mediators are of particular interest because they resist the rapid local enzymatic inactivation of these autacoids.^{12,15} Stable analogs, including 17(R/S)-methyl RvD1, were designed to mimic the aspirin-triggered form of RvD1 and were prepared by total organic synthesis to give potent and protective actions in ischemia-reperfusion injury; RvD1 and 17(R/S)-methyl RvD1 protected lungs from PMN infiltration and second organ injury (see Kasuga et al¹⁶ and the recent review by Serhan and Petasis¹⁷).

RvD1 and aspirin-triggered RvD1 (AT-RvD1) display similar actions, each reducing PMN infiltration by ~50% in the murine dorsal air pouch at equal doses of 100 ng per mouse. In murine peritonitis, RvD1 and AT-RvD1 also proved equipotent (at nanogram dosages), limiting PMN infiltration in a dose-dependent manner. They were as potent as indomethacin, a well-characterized nonsteroidal anti-inflammatory drug, in reducing PMN infiltration.^{11,12,18} In mouse kidneys, RvD1 is generated in response to bilateral ischemia-reperfusion injury.¹⁹ Administration of RvDs before or after ischemia results in a reduction in functional and morphologic kidney injury. RvD1 also displays potent ocular actions, reducing retinopathy²⁰ and suture-induced corneal neovascularization.²¹ As a potent resolution agonist, RvD1 also attenuates inflammatory pain.²² AT-RvD1 is antihyperalgesic in adjuvant-induced arthritis²³, and RvD1 and AT-RvD1 each block activities of specific transient receptor potential channels, attenuating acute pain behaviors and reversing mechanical and thermal hypersensitivity associated with inflammation.²⁴ Recently, AT-RvD1 proved to prevent experimental colitis.²⁵ In these studies, systemic treatment with AT-RvD1 (in nanogram ranges) greatly improved disease severity, including colonic damage, PMN infiltration, and body weight loss in dextran-sulfate-sodium- and 2,4,6-trinitrobenzene-sulfonic-acid-induced colitis. In obese diabetic mice, RvD1 improves insulin sensitivity and reduces macrophage accumulations in adipose tissues.²⁶ Hence, these results suggest that RvD1, AT-RvD1, and their stable analogs could provide a novel approach to stimulate resolution, treating a range of inflammatory disorders.

Two specific human GPCRs, ALX/FPR2 and GPR32, mediate RvD1's proresolving actions on human phagocytes.²⁷ Actions of RvD1 *in vivo* are mediated, in part, by molecular circuits involving microRNAs (miRNAs) miR-146b, miR-219, and miR-208a and their target genes, which are regulated by these receptors.²⁸ miRNAs are emerging as key regulators of the immune response and

inflammation-related diseases such as cancer.²⁹ Earlier, we identified the first miRNA signature of resolution as part of the molecular circuit that actively drives the return to homeostasis.²⁸ Herein, we determined the ligand selectivity of RvD1, its aspirin-triggered epimer AT-RvD1, and their stable analogs using the recently identified cognate receptors human GPR32 (hGPR32: DRV1) and human ALX/FPR2 (hALX/FPR2) and their ability to regulate PMN infiltration and specific miRNAs *in vivo*.

Materials and Methods

GPCR- β -Arrestin System and Ligand-Receptor Interactions

Ligand-receptor interactions were monitored using the β -arrestin PathHunter system (DiscoverRx, Fremont, CA)³⁰ and were performed essentially as in the study by Krishnamoorthy et al,²⁷ with CHO cells stably overexpressing recombinant hGPR32 (CHO-hGPR32) or HEK cells stably expressing hALX/FPR2 receptors (HEK-hALX/FPR2) tagged with the Pro-Link label of β -galactosidase and β -arrestin linked to the enzyme acceptor fragment of β -galactosidase. Briefly, specific ligand-receptor interactions on incubation with tested compounds (1 hour at 37°C) were determined by measuring chemiluminescence using the PathHunter detection kit (DiscoverRx). RvD1 and AT-RvD1 were prepared by total organic synthesis, and their physical and spectroscopic properties were matched with biogenic products using published criteria as reported in several studies.^{11,12,18} 17(R/S)-methyl RvD1 was also synthesized by total organic synthesis.¹⁶ Ligand-receptor interactions were monitored using HEK-hALX/FPR2 or CHO-hGPR32. Each compound was tested from 10⁻¹² to 10⁻⁷ mol/L. EC₅₀ values were calculated using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). For methyl ester preparation, RvD1 was treated with excess ethereal diazomethane, taken to dryness with nitrogen gas, suspended in methanol, and isolated using high-performance liquid chromatography. The physical integrity of RvD1 and each related compound was confirmed just before the bioassays.

Ligand Selectivity Using an ECIS System

Ligand-receptor interactions were monitored by measuring impedance³¹ across cultured CHO-hGPR32 cell monolayers using an electric cell-substrate impedance sensing (ECIS) system (Applied Biophysics, Troy, NY). Briefly, cells were plated at 0.1 × 10⁶ per well of an 8-well ECIS array (8W10E+). Cells were examined 24 hours after plating. RvD1 and related compounds were added to the chambers in serum-free medium, and real-time impedance changes were monitored (0 to 30 minutes, 37°C).

Genetically Engineered Mice, Peritonitis, and Fluorescence-Activated Cell Sorter Staining

Genetically engineered mice were produced and genotyped as reported earlier.^{32,33} All the animal experiments were performed in accordance with the Harvard Medical School Standing Committee on Animals guidelines for animal care (Protocol 02570). Briefly, mice were anesthetized with isoflurane (Aerrane; Baxter, Deerfield, IL) and were treated with either RvD1–methyl ester (RvD1-ME; 10 ng) or vehicle, i.v. in 100 μ L of sterile saline followed by i.p. with 1 mg of zymosan A in 1 mL of sterile saline. Twenty-four hours after injection, mice were euthanized with an overdose of isoflurane, and peritoneal exudates were collected to determine exudate leukocyte numbers and flow cytometry of PMN (with anti-Ly-6G antibody; eBioscience Inc., San Diego, CA) and monocytes/macrophages (with anti-F4/80 antibody; eBioscience).

miRNA Isolation, Reverse Transcription, and Real-Time PCR

miRNA isolation and real-time PCR were performed as in the study by Recchiuti et al.²⁸ Briefly, miRNA fractions from exudate cells collected from mice or human macrophages were isolated using an miRNA isolation kit (Roche Applied Science, Indianapolis, IN) and were quantitated using a spectrophotometer (NanoDrop, Thermo Fisher Scientific Inc., Wilmington, DE) at the Biopolymers Facility at Harvard Medical School (Boston, MA). miRNA samples were then reverse transcribed using the miScript reverse transcription kit (Qiagen Inc., Valencia, CA), followed by real-time PCR with specific primers for each miRNA in a SYBR green-based detection system. PCR was performed using an ABI7900HT thermal cycler (Applied Biosystems, Foster City, CA), and the results were analyzed using SDS software version 2.4 (Applied Biosystems). miRNA expression levels were calculated using the $2^{-\Delta\text{CT}}$ relative quantitation³⁴ after normalization to housekeeping genes using RNAU1A and small Cajal body-specific RNA 17 (SCARNA17, NCBI accession no. NR_003003) for mouse miRNA and the small nuclear RNA U1 (RNAU1A; NCBI accession no. NR_004421) for human macrophages.

Human Macrophages, Transfection, and IL-10 Production

Human monocytes were isolated by adherence from peripheral blood mononuclear cells²⁷ from deidentified healthy human volunteers from Children's Hospital Boston blood bank. The cells were cultured in RPMI supplemented with recombinant granulocyte-monocyte colony-stimulating factor (10 ng/mL) for macrophage differentiation. On day 7, macrophages were transfected with miR-208a expression vector or mock vector (OriGene Technologies Inc., Rockville, MD) using the jetPEI macrophage transfection reagent (Polyplus, Illkirch, France) with 5 μ g of expression vector per 2.5×10^6 cells. Seventy-two hours after transfection, miRNA was isolated and subjected to real-time PCR to verify overexpression of miR-208a. Supernatants

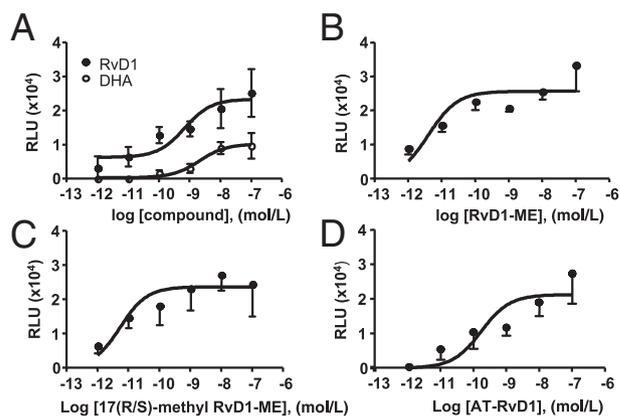


Figure 1. RvD1, AT-RvD1, and their stable analogs directly act on hALX/FPR2. Ligand-receptor interaction was monitored using the HEK-ALX/FPR2 β -arrestin system (see *Materials and Methods*). Dose-response activation curves were obtained for RvD1 and DHA (A), RvD1-ME (B), 17(R/S)-methyl RvD1-ME (C), and AT-RvD1 (D). Results are expressed as mean \pm SEM ($n = 3$ to 4). RLU, relative luminescence units.

were collected, and IL-10 levels were determined using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) and were normalized to total supernatant proteins measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

Statistical significance between two groups was evaluated using the 2-tailed Student's *t*-test. A $P < 0.05$ was considered significant. For determining significance among groups in the peritonitis experiment, one-way analysis of variance was used, and $P < 0.05$ across the groups was considered significant.

Results

RvD1, Its Aspirin-Triggered Epimer AT-RvD1, and Their Stable Analogs Directly Activate hALX/FPR2 and hGPR32

We first evaluated the activation of hGPR32 and hALX/FPR2 by AT-RvD1, RvD1-ME, 17(R/S)-methyl RvD1-ME and its precursor n-3 fatty acid DHA using the β -arrestin-based ligand-receptor interaction system.²⁷ Each of the related RvD1 products are potent bioactive analogs.^{12,16} At equimolar concentrations, RvD1, AT-RvD1, RvD1-ME, and 17(R/S)-methyl RvD1-ME directly activated hALX/FPR2 (Figure 1) and hGPR32 (Figure 2), with EC_{50} values in the low picomolar range (Table 1). In comparison, the RvD1 precursor DHA required log orders of magnitude higher concentrations to activate the tagged β -arrestin recruitment in these cells (Figures 1 and 2). At 10^{-9} mol/L, within the bioactive range of RvD1,^{11,12} DHA was far less effective (Figure 1A), displaying $\sim 10\%$ maximal activity of RvD1 with GPR32 and $\sim 50\%$ maximal activity with the hALX/FPR2 recombinant system.

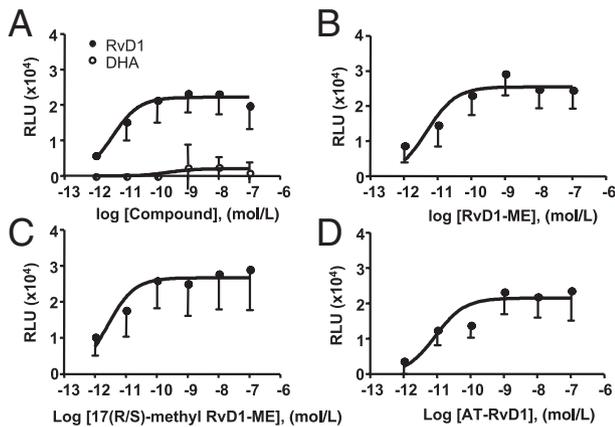


Figure 2. RvD1, AT-RvD1, and their stable analogs directly act on hGPR32. Ligand-receptor interactions were determined using the GPR32 β -arrestin system as in Figure 1. Dose-response activation curves are shown for RvD1 and DHA (A), RvD1-ME (B), 17(R/S)-methyl RvD1-ME (C), and AT-RvD1 (D). Results are expressed as mean \pm SEM ($n = 4$ to 6). RLU, relative luminescence units.

RvD1 and Compound 43 Evoke Rapid Receptor-Dependent Impedance Changes

To further examine ligand-receptor relationships, we used an ECIS system, which monitors changes in impedance on ligand binding to receptors.³¹ In this system, RvD1 dose dependently (ie, 1, 10, and 100 nmol/L) elicited rapid changes in impedance with CHO-hGPR32 β -arrestin cells (Figure 3A). The anti-inflammatory hALX/FPR2 agonist compound 43³⁵ also evoked dose-dependent changes in impedance with hGPR32-overexpressing cells similar to those elicited by RvD1 (Figure 3B). Equimolar concentrations of RvD1-ME, 17(R/S)-methyl RvD1-ME, and AT-RvD1 (Figure 3C) elicited similar changes in impedance with CHO-hGPR32 β -arrestin cells. Again, DHA was less effective (Figure 3D).

RvD1 Controls PMN Infiltration and Specific miRNAs in Genetically Engineered Mice

To assess *in vivo* and pinpoint RvD1 receptor-dependent actions, we tested RvD1-ME in zymosan-induced perito-

nititis with transgenic mice overexpressing hALX/FPR2. hALX/FPR2 transgene was placed under the control of a human CD11b promoter that drives high levels of transgene expression in mature murine myeloid cells.³² As shown in Figure 4, RvD1 (10 ng per mouse, i.v.) significantly reduced ($\sim 38\%$) the total peritoneal leukocyte count at 24 hours in nontransgenic littermates (mean \pm SD: $14.0 \pm 1.5 \times 10^6$ versus $22.6 \pm 3.0 \times 10^6$ in zymosan-treated mice; $P < 0.05$, one-way analysis of variance). In hALX/FPR2 transgenic mice, RvD1 administration resulted in a further decrease ($\sim 53\%$) in mean \pm SD total leukocyte numbers from $15.6 \pm 3.2 \times 10^6$ in zymosan alone to $7.3 \pm 2.1 \times 10^6$ ($P < 0.05$, one-way analysis of variance) (Figure 4). RvD1 reduced PMN numbers in hALX/FPR2 transgenic mice and nontransgenic littermates by 30% and 40%, respectively (Figure 4). With overexpression of hALX/FPR2, PMN numbers were $\sim 50\%$ lower than in nontransgenic littermates at 10 ng of RvD1 per mouse. In the absence of zymosan and RvD1, resident peritoneal cell numbers were not significantly different in ALX/FPR2 transgenic mice and their nontransgenic littermates (Figure 4).

RvD1 regulates miRNAs and their target genes associated with resolution of acute inflammation.²⁸ Since RvD1 activates hALX/FPR2, we next tested whether RvD1 can regulate some of these miRNAs during self-limited acute peritonitis in hALX/FPR2 transgenic mice. Small RNA fractions were isolated from peritoneal lavages collected 24 hours after injection of zymosan. RvD1 significantly up-regulated miR-208a by 1.7-fold ($P = 0.01$) (Figure 5A) and miR-219 by 1.8-fold ($P < 0.01$) (Figure 5B) compared with zymosan alone in hALX/FPR2 transgenic mice. RvD1 significantly down-regulated miR-208a levels by 0.4 fold ($P < 0.05$) in wild-type (WT) mice (Figure 5A) at 24 hours peritonitis, whereas it did not regulate miR-21, miR-146b, and miR-302d levels (Figure 5C). These results indicate that RvD1 regulates specific miRNAs endogenously expressed in resident peritoneal cells (Figure 5D), ie, miR-219 and miR-208a, in hALX/FPR2 transgenic mice, which also confirms those miRNA identified²⁸ as proresolving *in vivo*.

To further investigate ALX/FPR2-dependent actions of RvD1 *in vivo*, we next tested whether RvD1 could control

Table 1. EC₅₀ Values and Maximal Activity for RvD1 and Related Structures with Recombinant hALX/FPR2 and hGPR32 in a β -Arrestin Receptor System

Compound	EC ₅₀ , mean (95% CI) (mol/L)	RLU _{max} ($\times 10^4$)	% of max
hALX/FPR2			
RvD1	4.5×10^{-11} (4.7×10^{-12} to 4.4×10^{-10})	2.0	100
RvD1-ME	3.7×10^{-12} (3.3×10^{-13} to 4.0×10^{-11})	2.4	125
17(R/S)-methyl RvD1	5.4×10^{-12} (1.2×10^{-12} to 2.5×10^{-11})	2.3	104
AT-RvD1	1.8×10^{-10} (9.9×10^{-12} to 3.1×10^{-9})	2.1	115
DHA	1.9×10^{-9} (8.3×10^{-10} to 4.2×10^{-9})	1.0	50
hGPR32			
RvD1	3.6×10^{-12} (1.6×10^{-12} to 8.2×10^{-12})	1.1	100
RvD1-ME	4.6×10^{-12} (1.0×10^{-12} to 2.1×10^{-11})	1.3	116
17(R/S)-methyl RvD1	2.5×10^{-12} (7.8×10^{-13} to 8.1×10^{-12})	1.3	98
AT-RvD1	8.8×10^{-12} (1.5×10^{-12} to 5.0×10^{-11})	1.0	124
DHA	2.2×10^{-10} (2.0×10^{-12} to 2.5×10^{-12})	0.1	10

Ligand-receptor interactions were monitored using HEK-hALX/FPR2 or CHO-hGPR32, a β -arrestin cell system (see *Materials and Methods*). Percentage of maximal activity was calculated relative to the maximal RLU values of RvD1. Results are expressed as mean (95% CI, $n = 4$ to 6). CI, confidence interval; RLU, relative luminescence units.

leukocyte infiltration and regulate proresolving miRNAs in ALX/FPR2 receptor knockout mice, where LXA₄ did not regulate leukocyte trafficking.³³ As shown in Figure 6, A and B, RvD1 (10 ng per mouse, i.v.) significantly reduced zymosan-initiated leukocyte (by ~30%) and PMN (by ~27%) infiltration 24 hours after zymosan injection in WT mice. In ALX/FPR2^{-/-} mice, RvD1 did not regulate PMN infiltration (Figure 6). This finding is consistent with recent results with RvD1 in ALX/FPR2^{-/-} mice and leukocyte trafficking.³⁶ With zymosan alone, total leukocyte and PMN values were not significantly different between ALX/FPR2^{-/-} and WT mice. We also investigated the regulation of miR-219 and miR-208a by RvD1 in ALX/FPR2^{-/-} mice. As shown in Figure 6, C and D, in receptor-deficient mice, RvD1 did not significantly alter miR-208a or miR-

219 expression at 24 hours. In addition, RvD1 significantly enhanced IL-10 levels in WT mice that was lost in ALX/FPR2^{-/-} mice (Figure 7A). Taken together, these results indicate that RvD1 regulation of leukocyte trafficking, miR-208a, and IL-10 in mice depends on the endogenous ALX/FPR2 receptor.

Overexpression of miR-208a Up-Regulates IL-10 Secretion in Human Macrophages

miR-208a down-regulates mRNA levels of the proinflammatory PDCD4, a suppressor of IL-10 production³⁷ in human macrophages. Since RvD1 significantly up-regulated miR-208a in human macrophages overexpressing hALX/FPR2²⁸

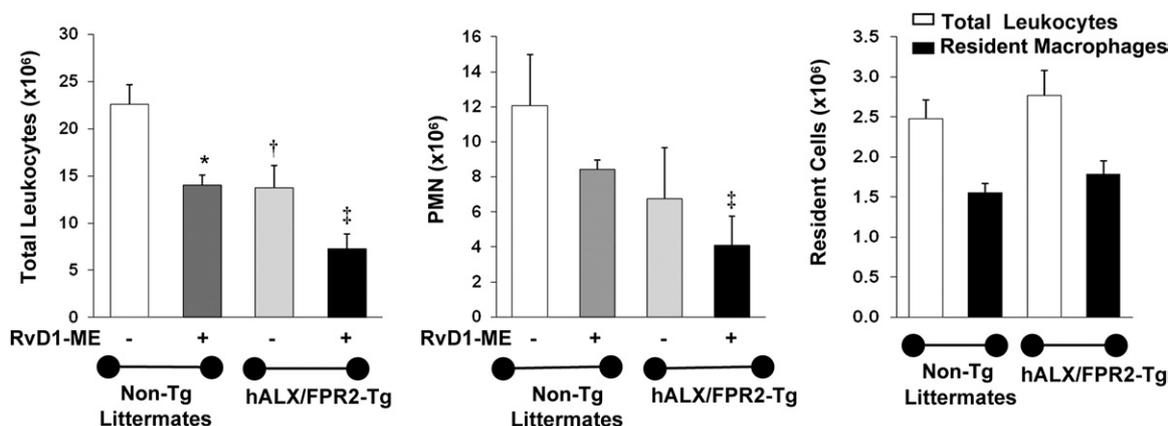


Figure 4. RvD1 reduction in PMN infiltration is enhanced in hALX/FPR2-overexpressing transgenic (Tg) mice peritonitis. hALX/FPR2-Tg mice and WT littermates were injected with zymosan (1 mg per mouse i.p.), with or without RvD1-ME at 10 ng per mouse i.v. Inflammatory exudates were collected at 24 hours and total exudate leukocytes were determined (see *Materials and Methods*): total leukocytes (left panel) and PMNs (middle panel). The right panel shows numbers of total leukocytes and resident macrophages in control mice. Results are expressed as mean \pm SEM ($n = 3$ mice). * $P < 0.05$, WT, zymosan versus zymosan + RvD1; † $P = 0.05$, zymosan, WT versus ALX/FPR2-Tg; ‡ $P < 0.01$, zymosan + RvD1 WT versus ALX/FPR2-Tg.

Figure 3. RvD1, Rv analogs, and compound 43 produce hGPR32-dependent changes in impedance. Ligand-receptor-dependent changes in impedance were continuously recorded with real-time monitoring across cell monolayers using an ECIS system with hGPR32-expressing β -arrestin cells. Results are tracings obtained from incubations with CHO-GPR32 β -arrestin cells plus RvD1 (1, 10, and 100 nmol/L) (A); compound 43 (1, 10, and 100 nmol/L) (B); 100 nmol/L RvD1, RvD1-ME, and 17(R/S)-methyl RvD1-ME (C); and 100 nmol/L RvD1 directly compared with native DHA (D). Each tracing is representative of $n = 3$. Insets in A and B show dose-dependent changes in cell impedance determined at 5 minutes of incubation of GPR32-overexpressing cells with RvD1 or compound 43 or vehicle alone. Results are expressed as mean \pm SEM ($n = 3$).

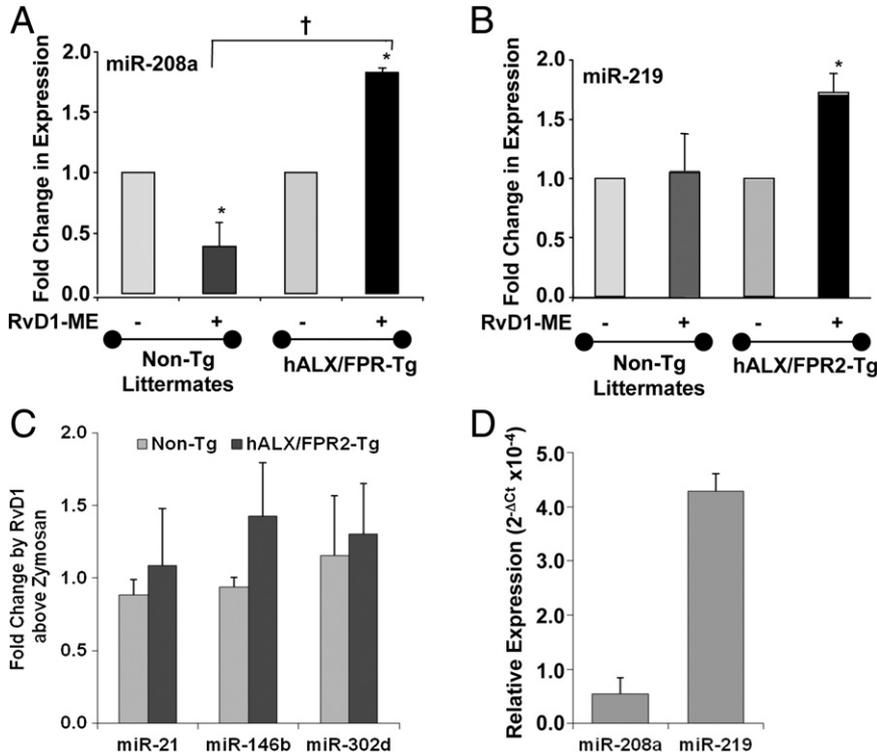


Figure 5. RvD1 stimulates specific proresolving miRNAs in hALX/FPR2 transgenic (Tg) mice. miRNA fractions were isolated from peritonitis exudate samples of WT littermates and ALX/FPR2-Tg mice challenged with zymosan (1 mg per mouse) compared with mice given RvD1 (10 ng per mouse with zymosan). Real-time PCR analyses of indicated miRNAs were performed and results were analyzed by the $2^{-\Delta C_T}$ method. Fold change in expression levels in RvD1-treated mice compared with zymosan alone were calculated from $2^{-\Delta C_T}$ values. Results are expressed as mean \pm SEM ($n = 3$ in each group) fold change in levels of miR-208a (A), miR-219 (B), and miR-21, miR-146b, and miR-302d (C). * $P < 0.05$ for RvD1-treated versus zymosan-treated WT or Tg mice; † $P < 0.05$ for zymosan + RvD1-treated Tg mice versus zymosan + RvD1-treated WT mice. D: miR-208a and miR-219 expression levels determined by real-time PCR in peritoneal cells from untreated mice.

and in hALX/FPR2 transgenic mice (*vide supra*), we sought to determine whether miR-208a directly regulates the anti-inflammatory cytokine IL-10. As shown in Figure 7B, overexpression of miR-208a in human macrophages resulted in a significant increase in released IL-10 levels compared with mock transfected macrophages.

Discussion

In the present study, we determined the ligand selectivity of the novel proresolving chemical mediator RvD1 and related structures with two recently identified GPCRs, hGPR32 and hALX/FPR2, that are activated by RvD1.

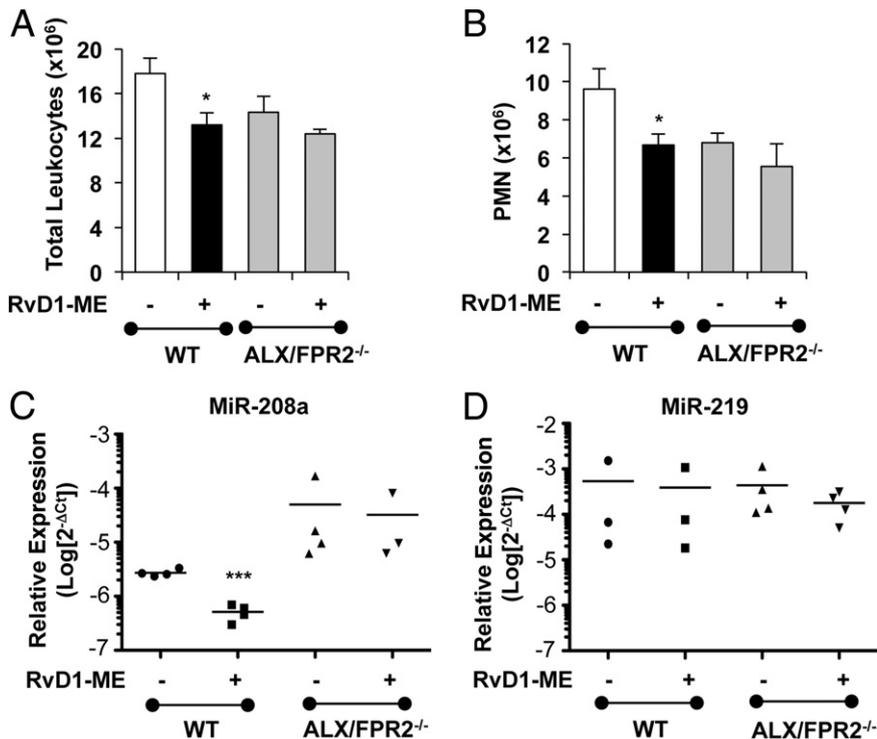


Figure 6. Regulation of acute inflammation and miRNA by RvD1 in ALX/FPR2 knockout mice. Number of total leukocytes (A) and PMNs (B) in peritoneal lavage fluid from WT or ALX/FPR2^{-/-} mice injected with zymosan (1 mg per mouse, i.p.) with or without RvD1-ME (10 ng per mouse, i.v.). Lavage fluid samples were collected 24 hours after initiation of peritonitis, and cells were stained with anti-Ly-6G and F4/80 antibodies. Results are expressed as mean \pm SEM ($n = 6$ mice per group). * $P < 0.05$ versus zymosan-treated group. Expression of miR-208a (C) and miR-219 (D) in exudate cells from WT or ALX/FPR2^{-/-} mice 24 hours after injection of zymosan plus RvD1-ME (10 ng per mouse, i.v.) or zymosan plus saline. Relative expression of miRNAs was determined using real-time PCR. Results are expressed as mean \pm SEM of fold changes in expression ($n = 6$ mice per group). *** $P < 0.001$ versus zymosan-treated group.

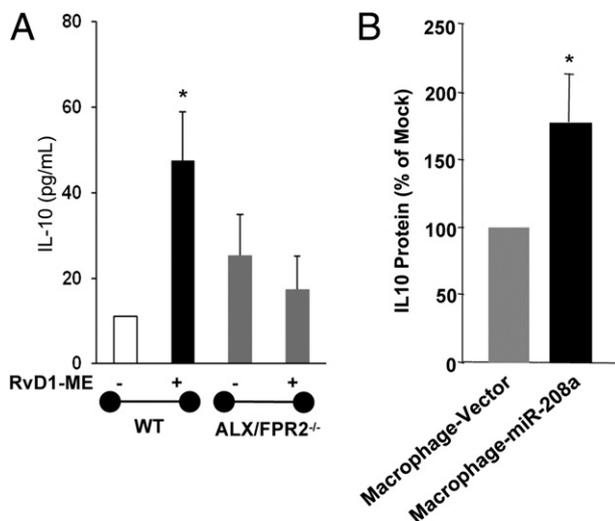


Figure 7. Regulation of IL-10 levels by RvD1 in peritonitis and by miR-208a in human macrophages. **A:** IL-10 levels were determined in hALX/FPR2^{-/-} mice ($n = 3$ to 6). * $P < 0.05$ versus mice receiving zymosan alone. **B:** IL-10 levels were determined in human macrophages transiently overexpressing miR-208a or control vector ($n = 3$). * $P < 0.05$ versus control vector-transfected macrophages.

Results from these analyses demonstrate that RvD1-hALX/FPR2 interactions regulate acute inflammatory responses *in vivo* and selected miRNA expression in hALX/FPR2 transgenic mice to reduce inflammation. Along these lines, an RvD1-regulated miR-208a identified in hALX/FPR2 transgenic mice also controlled release of the anti-inflammatory cytokine IL-10 in human macrophages.

We examined RvD1, AT-RvD1, and their analogs in hALX/FPR2 and hGPR32 receptor-overexpressing cells using β -arrestin reporter systems to access ligand potencies since each is known to display potent actions *in vivo* in a range of animal models (*vide supra*).³ In these experiments, the RvD1 precursor DHA proved to be less active with these receptors (Figures 1–3). Ligand-receptor-stimulated changes were also monitored with electrode-based impedance measurements, which reflect changes in the actin cytoskeletal framework.³⁸ Because RvD1 regulates actin cytoskeletal changes,²⁷ we sought to use this system where these changes reflect GPCR activation. As shown in Figure 3, A and B, RvD1 and the anti-inflammatory compound 43³⁵ each elicited dose-dependent changes in impedance in hGPR32-overexpressing CHO cells. Similarly, RvD1, AT-RvD1, and their analogs evoked rapid changes in impedance at equimolar concentrations (Figure 3, C and D) with these cells, indicating that activation of this receptor is consistent with the bioactivity of RvD1 and related structures. These results also indicate that in the 17S-containing RvD1 and the 17R- as in the aspirin-triggered epimer AT-RvD1, each configuration at carbon position C17, ie, C17 alcohol groups, is efficiently recognized by receptors hGPR32 and hALX/FPR2. RvD1 showed a lower EC₅₀ for the hALX/FPR2 receptor in the β -arrestin system, suggesting that in these conditions, the S configuration of the C17 hydroxyl group is preferred for interactions with hALX/FPR2 receptor. Also, the addition of a carboxyl methyl ester group to RvD1 (denoted RvD1-ME) retained its abil-

ity to activate with these receptors. In contrast, the native DHA structure that lacks functional alcohol groups was far less effective than was RvD1 at activating these receptors within the active concentration/dose range of RvD1 and closely related structures. Although DHA at 10⁻⁹ mol/L can partially activate ALX/FPR2, this seems to be relatively weak compared with RvD1 (Table 1). RvD1 and its stable analogs were 2 log orders of magnitude more potent (EC₅₀ ~10⁻¹² to 10⁻¹¹ mol/L). In this regard, RvD1 is present in human plasma at ~5 × 10⁻¹¹ mol/L,³⁹ consistent with its agonist actions.

Earlier, we demonstrated, using a single cell-monitoring microfluidic chamber, the specific PMN actions of RvD1 at nanomolar levels and its direct actions with human PMN that clearly require conversion of DHA, the precursor of RvD1, to stimulate actions on human PMN.¹⁶ Thus, the present results also confirm that specific actions of RvD1 and its analogs are mediated via interactions with surface receptors. Because RvD1 interacts with hALX/FPR2,²⁷ we also tested whether RvD1 can dampen inflammation in hALX/FPR2 transgenic mice. As shown in Figure 4, RvD1 as low as 10 ng i.v. decreased total leukocyte infiltration in zymosan-induced murine peritonitis in WT mice. In parallel, 10 ng of RvD1 significantly reduced total leukocyte and PMN numbers in hALX/FPR2 mice compared with zymosan alone. These results indicate that RvD1 signals via ALX/FPR2 *in vivo* for its anti-inflammatory actions. Of interest, RvD1 did not change the expression of ALX/FPR2 in peritoneal exudate cells from WT mice, which was determined by quantitative real-time PCR analyses (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). hALX/FPR2 was initially identified as the receptor for LXA₄⁴ and was later shown to be activated by aspirin-triggered lipoxin (15-epimer of LXA₄),⁴⁰ the glucocorticoid-derived annexin peptide,⁴¹ and RvD1²⁷ and by many proinflammatory peptides (see reviews^{1,42}). Each of these proresolving mediators interacting with hALX/FPR2 signals for anti-inflammatory and proresolving actions. By comparison, as low as 10 ng of aspirin-triggered lipoxin per mouse blocked zymosan-stimulated PMN infiltration by 50% in hALX/FPR2 transgenic mice compared with in WT littermates.³²

In the present study, we also assessed the anti-inflammatory actions of an ALX/FPR2 agonist, compound 43 (see Supplemental Figure S2 at <http://ajp.amjpathol.org>), that was identified by a medical chemical screening approach.³⁵ In Supplemental Figure S2 (available at <http://ajp.amjpathol.org>), this receptor agonist, compound C43, blocked total leukocyte and PMN infiltration *in vivo* in murine zymosan peritonitis, confirming its anti-inflammatory actions. Recently, it was reported that targeted knockdown of the murine ALX/FPR2 receptor diminished the anti-PMN activities of its ligands, namely, LXA₄, annexin peptide, and compound 43, and gave an exacerbated inflammatory phenotype in mice.³³ In these ALX/FPR2-null mice, RvD1's protective action (ie, reducing peritoneal PMN infiltration at 4 hours with 1 and 10 ng per mouse) was also diminished, indicating the crucial role of ALX/FPR2 expression in RvD1 biology in the mouse.³⁶ Along these lines, low-dose aspirin in humans is protective and anti-inflammatory in a cantharidin-induced acute

skin inflammation model. These actions of aspirin depended on the endogenous biosynthesis of 15-epimer of LXA₄ in skin blisters and on the up-regulation of hALX/FPR2 expression in peripheral blood leukocytes.⁴³ Also, the amplitude and time course of 15-epimer of LXA₄ and hALX/FPR2 appearance were key determinants in the severity of inflammation and the onset of resolution in these individuals,¹⁰ thus unveiling in humans the important role of hALX/FPR2 and its agonists in governing host responses. Recently, it was shown that ALX/FPR2 expression is up-regulated during the menstrual phase of the cycle and in decidua tissue from the first trimester of pregnancy. LXA₄ reduces the inflammatory cytokines in human endometrium and decidua tissue, indicating that the LXA₄-ALX/FPR2 axis regulates inflammatory responses in human endometrium and decidua of early pregnancy.⁴⁴ Hence, it would not be surprising if the ligand-receptor interactions documented in the present study can also be of interest in other organ systems in addition to the immune response and its endogenous resolution mechanisms.

In the effector immune system, certain GPCRs can be activated by bias ligands that couple different cellular responses, as in the case of ALX/FPR2, which interacts with peptides and lipid mediators that function as proinflammatory or anti-inflammatory and proresolving.^{8,42} This also seems relevant for the resolvin E1 receptor ChemR23, which binds both peptides and is stereoselective for RvE1 and its proresolving actions.⁴⁵ In view of these findings, it is possible that proresolving ligands use receptors that play a role in initiation and in resolution and termination of the inflammatory response to evoke novel tissue signals required for resolution and homeostasis. RvD1 acts via specific miRNAs that control the inflammatory response, defining the first RvD1-GPCR miRNA signature of resolution in inflammatory exudates.²⁸ To further address these novel proresolving mechanisms in the present experiments, we also assessed regulation of these key miRNAs by RvD1 *in vivo* using transgenic mice that overexpress the human receptor for LXA₄, hALX/FPR2. Among the analyzed miRNAs, RvD1 given at 10 ng per mouse *i.v.* selectively up-regulated miR-208a and miR-219 in hALX/FPR2 transgenic mice at 24 hours of peritonitis compared with those challenged with zymosan alone or with their nontransgenic littermates. In comparison, RvD1 did not up-regulate either miR-208a or one of its target gene products, IL-10, in genetically modified ALX/FPR2 knockout mice. This finding in receptor-deficient mice further corroborates the evidence that *in vivo* actions of RvD1 are mediated, at least in part, by the ALX/FPR2 receptor, which, in turn, regulates proresolving miRNAs and target genes. In human macrophages, miR-208a and miR-219 were identified as RvD1-GPCR-regulated miRNAs along with miR-146b and miR-21. These miRNAs were also regulated by RvD1 in murine peritoneal exudate leukocytes. These miRNAs and their target genes seem to belong to an integral network of regulatory molecules that promote resolution of acute inflammation.²⁸ In the present experiments, miR-208a is down-regulated by RvD1 in WT littermates and is up-regulated in hALX/FPR2-overexpressing receptor transgenic mice.

These findings indicate the existence of a specific RvD1 receptor-dependent circuit that is required for the regulation of miR-208a. These results also suggest that human and mouse ALX/FPR2 regulate miR-208a *in vivo* differently.

Recently, we found that RvD1 up-regulates miR-208a and reduces miR-219 expression in human macrophages overexpressing hGPR32, which was not the case for human ALX. RvD1 with human ALX overexpression did not increase miR-208a in human macrophages,²⁸ whereas overexpression of hALX/FPR in transgenic mice (Figure 5A) gave an increase in miR-208a when RvD1 was administered. Hence, the direct actions of RvD1 with isolated human macrophage overexpression of ALX²⁸ versus *in vivo* administration of RvD1, as in the present results (Figure 5A), give different levels of miR-208a. This difference with miR-208a might reflect the *in vivo* inflammatory exudate environment or possibly species differences with RvD1 postreceptor signal transduction. Also, it is possible that miR-208a regulation *in vivo* in mice could reflect its regulation via macrophages and other inflammatory exudate cells.

As for their target genes, miR-219 targets 5-lipoxygenase and regulates leukotriene B₄ production.²⁸ miR-208a overexpression in human macrophages increased IL-10 (Figure 7). miR-208a specifically targets and down-regulates PDCD4, a proinflammatory regulatory protein that reduces IL-10 and promotes activation of the NF-κB pathway.^{28,37} The present experiments with RvD1 are consistent with this and demonstrate that miR-208a overexpression in human macrophages up-regulates IL-10. This is likely important because IL-10 is a pivotal cytokine in inflammation and its resolution. Hence, IL-10 regulation by a local mediator, namely, RvD1, that is biosynthesized from an essential dietary ω-3 fatty acid may have wide-ranging implications in human health and in diseases such as cardiovascular disease (recently reviewed by De Caterina⁴⁶). Unlike human macrophages, the down-regulation of miR-208a by RvD1 was not associated with concurrent increases in PDCD4 levels in mice.²⁸ This finding suggests that PDCD4 might not be a direct target of miR-208a in murine peritoneal exudate cells. Indeed, it is also known that expression patterns and functions of miRNAs are not strictly conserved between species.^{47,48} This may also explain, in part, the increases in IL-10 levels in WT mice in response to RvD1 (Figure 7), albeit decreases in miR-208a levels in mice. Along these lines, IL-10 is expressed in several cell types and, hence, may have cell-specific transcriptional and posttranscriptional regulatory mechanisms.⁴⁹ Since RvD1 up-regulated IL-10 levels in peritoneal exudate cells, it is possible that RvD1 may use additional mechanisms in these cells that are independent of miR-208a to regulate IL-10 production and secretion.

Along these lines, the original complete structural elucidation of the first bioactive coined RvE1 relied on its ability to reduce PMN infiltration and proinflammatory mediators and to evoke the nonphlogistic activation of monocytes/macrophages.^{45,50} In more aggressive disease models associated with inflammation, infection, and tissue destruction, such as TNBS-induced mouse coli-

tis,⁵¹ ocular diseases,^{20,21} or even an infection-initiated chronic inflammation, as in rabbit periodontal disease,⁵² low doses of RvE1 proved tissue protective and in certain settings stimulated regeneration of the local tissues injured during PMN-mediated inflammatory responses. In support of this proresolving mechanism being tissue protective, RvD2 and AT-RvD1 were recently demonstrated by Bento et al²⁵ to be potent protective mediators in TNBS and DDS colitis in murine systems with AT-RvD1 relying on ALX/FPR2 for their protective actions in murine tissues. Hence, by activating different proresolving receptors and tissue circuits, specific proresolving mediators, such as LXA₄, RvE1, and RvD1 as ligands, each biosynthesized locally from different substrates, regulate leukocyte functional responses and stimulate tissue resolution.

In summation, the potent proresolving mediator RvD1, its aspirin-triggered epimer AT-RvD1, and the synthetic analogs RvD1-ME and 17(R/S)-methyl RvD1 each selectively activates recombinant hALX/FPR2 and hGPR32 receptors. RvD1 dampens acute inflammation *in vivo* in part via the hALX/FPR2 receptor, as demonstrated in transgenic mice *in situ*. In peritoneal exudate leukocytes from these mice, RvD1 up-regulates miR-208a, which targets PDCD4, a proinflammatory signaling molecule that gives up-regulation of IL-10 in human macrophages. Together, the findings of the present study establish the ligand selectivity for proresolving agonists of hALX/FPR2 and hGPR32 and indicate that RvD1 via its interactions with these specific GPCRs regulates miRNAs that target specific resolvers. They also shed light on new GPCR-dependent mechanisms for RvD1 and related structures and their actions in controlling the magnitude of the inflammatory response and stimulating its resolution.

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