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Accessibility
Lipoxin A<sub>4</sub> Counter-regulates Histamine-stimulated Glycoconjugate Secretion in Conjunctival Goblet Cells

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Conjunctival goblet cells synthesize and secrete mucins which play an important role in protecting the ocular surface. Pro-resolution mediators, such as lipoxin A<sub>4</sub> (LXA<sub>4</sub>), are produced during inflammation returning the tissue to homeostasis and are also produced in non-inflamed tissues. The purpose of this study was to determine the actions of LXA<sub>4</sub> on cultured human conjunctival goblet cell mucin secretion and increase in intracellular [Ca<sup>2+</sup>]<sub>i</sub> and on histamine-stimulated responses. LXA<sub>4</sub> increased mucin secretion and [Ca<sup>2+</sup>]<sub>i</sub>, and activated ERK1/2 in human goblet cells. Addition of LXA<sub>4</sub> before resolvin D1 (RvD1) decreased RvD1 responses though RvD1 did not block LXA<sub>4</sub> responses. LXA<sub>4</sub> inhibited histamine-stimulated increases in mucin secretion, [Ca<sup>2+</sup>]<sub>i</sub>, and ERK1/2 activation through activation of β-adrenergic receptor kinase 1. We conclude that conjunctival goblet cells respond to LXA<sub>4</sub> through the ALX/FPR2 receptor to maintain homeostasis of the ocular surface and regulate histamine responses and could provide a new therapeutic approach for allergic conjunctivitis and dry eye diseases.
debris. Recent evidence suggests that LXs and Rvs also play a role under normal, physiological conditions. In conjunctival goblet cells, RvD1 and its epimer aspirin-triggered RvD1 (AT-RvD1), and RvE1 appear to have two functions (1) alone these compounds increase [Ca^{2+}], activate extracellular regulated kinase (ERK) 1/2, and stimulate mucin secretion and (2) block LTD4 and histamine-stimulated increase in [Ca^{2+}], and mucin secretion.

LXA4 and lipoxin B4 (LXB4) are both biosynthesized from arachidonic acid. LXA4 binds to the ALX/FPR2 receptor causing a conformational change leading to stimulation of pro-resolution pathways. Similar to RvD1 and AT-RvD1, LXA4 and LXB4, and several stable analogs of LXA4 alone increased [Ca^{2+}], in conjunctival goblet cells from rats. LXA4 also increased mucin secretion utilizing the signaling pathways of phospholipase C, -D, and AK. The increase in [Ca^{2+}], stimulated by LXA4 was directly linked to mucin secretion as chelation of intracellular Ca^{2+} blocked secretion. In the present study, we investigated the actions of LXA4 with cultured human conjunctival goblet cells, as well as the impact of LXA4 on histamine-stimulated increase in [Ca^{2+}], mucin secretion, and ERK 1/2 activation in rat and human goblet cells. In human goblet cells, LXA4 binds to the ALX/FPR2 and GPR32 receptors while RvD1 binds to GPR32 receptors. In rat, LXA4 and RvD1 preferentially bind to the ALX/FPR2 receptor. In addition, we report that LXA4 utilizes β-adrenergic receptor kinase (βARK) 1 to counter-regulate the H1 histamine receptor.

Results
Action of LXA4 on [Ca^{2+}], and Protein Secretion in Human Goblet Cells. We previously demonstrated that LXA4 stimulated an increase in [Ca^{2+}], and mucin secretion in rat goblet cells. To investigate the actions of LXA4 on goblet cells grown from human conjunctiva, cells were stimulated with LXA4 from 10^{-11} M – 10^{-8} M and the increase in [Ca^{2+}], was measured. Pseudo colored images of goblet cells treated with increasing concentrations of LXA4 are shown in Fig. 1A. LXA4 increased [Ca^{2+}], in a concentration dependent manner with increases in peak [Ca^{2+}], of 97.6 ± 31.2, 762.0 ± 259.4, and 50.0 ± 14.4 nM at 10^{-11}, 10^{-10}, and 10^{-9} M, respectively (Fig. 1B,D). The increase at 10^{-10} M LXA4 was increased above basal (p=0.03).

LXA4 has been shown to bind to the ALX/FPR2 receptor in goblet cells isolated from rat conjunctiva. To determine whether LXA4 acts via ALX/FPR2 receptors in human goblet cells, goblet cells were pretreated with the ALX/FPR2 antagonist BOC-2 (10^{-4} M) for 30 min, prior to addition of LXA4 (10^{-10} M). Similar to rat goblet cells, in cultured human cells BOC-2 inhibited LXA4-stimulated increase in [Ca^{2+}], at 10^{-10} M by 98.5 ± 1.1% to 13.3 ± 11.7 nM (p=0.05) (Fig. 1C,D).

Next, the actions of LXA4 on glycoprotein secretion from cultured human goblet cells was determined. Goblet cells were serum-starved for 2 h and LXA4 was added (10^{-10}–10^{-8} M) for 2 h and glycoconjugate secretion measured. LXA4 (10^{-8} M) increased secretion 2.6 ± 0.1 fold above basal (p=0.01, Fig. 2). In cells from the same individuals, histamine, as a positive control, increased glycoconjugate secretion 2.5 ± 0.3 fold above basal (p=0.005, data not shown). These data show that in human goblet cells, similar to rat goblet cells, LXA4 activates the ALX/FPR2 receptor to increase [Ca^{2+}], and stimulate glycoconjugate secretion.

Presence and Localization of ALX/FPR2 Receptors in Human Conjunctival Goblet Cells. As LXA4 stimulates an increase in [Ca^{2+}], and glycoconjugate secretion and the ALX/FPR2 receptor inhibitor, BOC-2, blocks LX4-stimulated increase in [Ca^{2+}], we confirmed that ALX/FPR2 is expressed in human goblet cells. RT-PCR, using primers specific for this receptor, was performed. As shown in Fig. 3A, one band at the expected size was detected. The receptor is also expressed at the protein level as detected by western blot analysis from cells grown from 3 individuals (Fig. 3B). The ALX/FPR2 receptor is known to be glycosylated which could account for the multiple bands observed. In cultured human goblet cells, ALX/FPR2 (shown in red) was present throughout the cytosol of the cells (Fig. 3C). UEA-1, shown in green was used to confirm the identity of cultured goblet cells (Fig. 3C). There was substantial overlap in the localization of ALX/FPR2 and UEA-1. These data confirm that ALX/FPR2 is present in human goblet cells.

Interaction of LXA4 and RvD1 via ALX/FPR2 and GPR32. The ALX/FPR2 receptor has multiple agonists which can bind to it. These agonists include RvD1, the protein annexin A1, as well as LXA4. In addition to ALX/FPR2, RvD1 and LXA4, also bind to the receptor GPR32, which we previously demonstrated to be present in cultured human goblet cells. We explored the interaction between LXA4 and RvD1 with ALX/FPR2 and GPR32 in human goblet cells. In a first set of experiments, we determined the extent to which RvD1 binds to ALX/FPR2 in human goblet cells. Goblet cells were preincubated with ALX/FPR2 inhibitor BOC-2 (10^{-4} M) prior to stimulation with RvD1 (10^{-6} M) and [Ca^{2+}], was measured. In the absence of inhibitor, RvD1 increased [Ca^{2+}], by 242.8 ± 70.8 nM (Fig. 4A). Preincubation with BOC-2 had no effect on the RvD1 response (p=0.14).

In a second set of experiments, the following experimental paradigm was used: addition of first agonist was followed 5 minutes later by addition of second agonist and the increase in peak [Ca^{2+}], was measured approximately 30 seconds after addition of agonist. In cultured human goblet cells, the addition of RvD1 (10^{-8} M) first caused a peak increase in [Ca^{2+}], of 256.2 ± 62.5 nM (Fig. 4B,C). A second addition of RvD1, 5 minutes after the first, resulted in a peak increase of [Ca^{2+}], of 17.9 ± 13.8 nM (Fig. 4B,C). This was a decrease from the response obtained when RvD1 was added first (p=0.04). If LXA4 is added first and RvD1 is added 5 min later, the RvD1 response was 11.3 ± 11.3 nM. This is also a decrease from the response obtained if RvD1 is added first (p=0.03).

If LXA4 (10^{-8} M) is added first, the initial response obtained was a change in peak [Ca^{2+}], of 576.5 ± 149.8 nM (Fig. 4B,D). A second addition of LXA4 resulted in a decrease from the first peak and was 20.4 ± 13.8 nM (p=0.04). If RvD1 is given first, the addition of LXA4 resulted in change in peak [Ca^{2+}], of 391.7 ± 53.7 nM. This was not different from the result obtained with LXA4 added first (p=0.4, Fig. 4B,D) though it is increased from basal (p=0.002). The data shown in Fig. 4C,D are from the same individuals.
These data indicate that in human goblet cells, RvD1 preferentially binds to GPR32 which desensitizes the receptor to a second addition of RvD1 while the LXA4 response is not altered. In contrast, LXA4 binds to both the ALX/FPR2 and GPR32 receptor and a second addition of LXA4 desensitizes both receptors to a subsequent addition of either LXA4 or RvD1.

LXA4 Inhibits Histamine-stimulated Increase in Glycoconjugate Secretion, \([\text{Ca}^{2+}]_i\), and ERK Activation. To examine the effects of LXA4 on histamine-stimulated glycoconjugate mucin secretion, rat goblet cells were pretreated with LXA4 \((10^{-10} - 10^{-9} \text{M})\) for 30 min and stimulated with histamine \((10^{-5} \text{M})\) for 2 h.

Figure 1. Lipoxin A4 (LXA4) Increases Intracellular \([\text{Ca}^{2+}]_i\) \((\text{Ca}^{2+})_i\) in Human Conjunctival Goblet Cells. Pseudo-color images of goblet cells stimulated with increasing concentrations of LXA4 \((10^{-11} - 10^{-9} \text{M})\) is shown in (A). Effect of LXA4 (black line, LXA4 \(10^{-11} \text{M}\); red line, LXA4 \(10^{-10} \text{M}\); green line, LXA4 \(10^{-9} \text{M}\) on \([\text{Ca}^{2+}]_i\) over time is shown in (B) Goblet cells were preincubated with BOC-2 \((10^{-4} \text{M})\) for 30 min prior to addition of LXA4 and [Ca2+] was measured. Effect over time with LXA4 \(10^{-10} \text{M}\) is shown in (C) Change in peak [Ca2+] was determined (closed triangles) and is shown in (D). Change in peak [Ca2+] was calculated and is shown in (D). Data are either the mean of the response over time from 3 individuals (B) or individual values or mean ± SD from the same 3 individuals (D).
In untreated rat cells, histamine increased mucin secretion 1.9 ± 0.1 fold increase above basal (p = 0.002). LXA₄ blocked histamine-stimulated secretion by 75.8 ± 8.8 and 90.8 ± 4.0% at 3 × 10⁻¹⁰ and 10⁻⁹ M, respectively (p = 0.001 and 0.00002, Fig. 5A). In human goblet cells, histamine increased mucin secretion 2.5 ± 0.3 fold increase above basal (p = 0.005). Preincubation with LXA₄ 10⁻¹⁰ and 10⁻⁹ M blocked histamine-stimulated secretion by 85.5 ± 14.5 and 87.3 ± 8.4%, respectively (p = 0.004 and 0.0003, Fig. 5B).

We previously established that histamine increases [Ca²⁺], in a concentration-dependent manner which was blocked by RvD1 and AT-RvD1. To determine if LXA₄ also blocks histamine responses, cultured goblet cells were preincubated with LXA₄ prior to stimulation with histamine. In goblet cells cultured from rat, histamine (10⁻⁵ M) increased [Ca²⁺] with a peak of 587.1 ± 92.3 nM (p = 0.0002, Fig. 6A). Preincubation with LXA₄ (10⁻¹⁰–10⁻⁸ M) decreased histamine-stimulated increase in [Ca²⁺] at 10⁻⁹ and 10⁻⁸ M with maximum inhibition.
occurring at 10^{-9} M LXA4 which decreased the histamine response by 64.1 \pm 14.1\% to 185.6 \pm 52.1 nM (p = 0.01, Fig. 6A,B).

Similar results were obtained with cultured human goblet cells. Histamine (10^{-5} M) stimulated an increase in peak [Ca^{2+}]_i 1152.5 \pm 173.9 nM (p = 0.006, Fig. 6C,D). Preincubation with LXA_4 (10^{-10}–10^{-8} M) decreased histamine-stimulated increase in [Ca^{2+}]_i at all concentrations with maximum inhibition occurring at 10^{-10} M LXA_4 which decreased the histamine response by 66.1 \pm 7.3\% to 378.6 \pm 92.6 nM (p = 0.005, Fig. 6C,D).

To ensure that the actions of LXA_4 on histamine response are mediated by the ALX/FPR2 receptor, rat goblet cells were pretreated with BOC-2 (10^{-4} M) for 15 min prior to addition of LXA_4 (10^{-9} M) for 30 min. The [Ca^{2+}]_i was then measured in response to histamine (10^{-5} M). In the absence of BOC-2 and LXA_4, the change in peak [Ca^{2+}]_i in response to histamine was 586.8 \pm 173.1 nM (p = 0.01, Fig. 6E). LXA_4 added first reduced the histamine response by 90.3 \pm 1.9\% to 55.5 \pm 8.5 nM (p = 0.03). Preincubation with BOC-2 reversed the inhibition by LXA_4 on the histamine response and increased [Ca^{2+}]_i by 495.0 \pm 76.6 nM (Fig. 6E).

Histamine activates ERK 1/2 to stimulate glycoconjugate secretion which was also blocked by RvD1 and AT-RvD1^{15,20}. To determine if LXA_4 also blocks histamine-stimulated ERK 1/2 activity, rat goblet cells were preincubated with LXA_4 (10^{-10}–10^{-8} M) for 30 min prior to incubation with histamine (10^{-6} M) for 5 min and ERK 1/2 activity was measured. Histamine increased ERK 1/2 activity 1.3 \pm 0.1 fold increase above basal (p = 0.04, Fig. 7A,B). LXA_4 decreased this response at all concentrations (Fig. 7A). When four independent experiments were analyzed, inhibition with LXA_4 10^{-8} M decreased histamine response by 64.4 \pm 19.4\% to 1.1 \pm 0.1 fold increase above basal (p = 0.002, Fig. 7B).

**ALX/FPR2 Uses \beta ARK1, but Not Protein Kinase C, to Block the H1 Histamine Receptor Simulated Increase in [Ca^{2+}]_i.** Examination of the H1 histamine receptor for phosphorylation sites using Scan Site (http://scansite.mit.edu/), showed that this receptor has consensus sequences for \beta-adrenergic receptor kinase 1 (\beta ARK1), also known as G-protein coupled receptor kinase (GRK)-2 and protein kinase C (PKC). We previously demonstrated that RvD1 binding to GPR32 activates both these kinases to counter-regulate the H1 histamine receptor to block the increase in [Ca^{2+}]_i^{10}. To determine if ALX/FPR2 and LXA_4 also use \beta ARK1 and/
or PKC to counter regulate histamine H1 receptor, rat goblet cells were pretreated with either LXA₄ or LXA₄ plus inhibitors to βARK1 and PKC. The increase in [Ca²⁺]i, in response to the specific H1 receptor agonist, histamine dimaleate was measured in cultured rat goblet cells. Pretreatment with LXA₄ decreased the histamine dimaleate stimulated increase in [Ca²⁺]i from 823.7 ± 154.1 nM above basal in the absence of LXA₄ to 140.9 ± 68.7 nM (p = 0.02, Fig. 8A,B). βARK1 inhibitor peptide (10⁻⁶ M) alone did not have an effect of the histamine dimaleate (p = 0.16, Fig. 8A,B). When cells were pretreated with βARK1 inhibitor peptide followed by LXA₄, blockage of the histamine dimaleate response by LXA₄ was completely reversed (Fig. 8A,B).

To investigate the role of PKC in counter-regulation of H1 receptor by LXA₄, rat goblet cells were preincubated with the PKC inhibitor Ro317549 (10⁻⁷ M). In these experiments, histamine dimaleate increased [Ca²⁺]i by 751.7 (p = 0.01, ± 216.1 nM (Fig. 8C,D). This response was decreased by LXA₄, and was 193.3 ± 11.3 nM (p = 0.04, Fig. 8C,D). Ro317549 alone had no effect on histamine dimaleate response (Fig. 8C,D). Addition of the PKC inhibitor prior to LXA₄ had no effect on the LXA₄ blockage of histamine dimaleate (Fig. 8C,D).

These data indicate that the activation of the ALX/FPR2 uses βARK1 but not PKC to counter regulate the H1 histamine receptor in rat goblet cells. This is in contrast to RvD1 that uses both βARK1 and PKC to counter regulate the H1 receptor.

Discussion

Our results demonstrate that LXA₄ plays a role in goblet cell function in both normal non-inflamed conditions and acute inflammatory conditions. Our hypothesis is that the ALX/FPR2 receptor is present in human conjunctival goblet cells and activation of the receptor by LXA₄ stimulates an increase in [Ca²⁺], and mucin secretion, which in rat goblet cells is protective in the eye and involves activation of phospholipase (PL) C, PLD, and PLA2 signaling pathways (Fig. 9A)19. In circumstances such as inflammation or pharmacological addition, LXA₄ inhibits histamine-stimulated increase in [Ca²⁺], ERK 1/2 activation, and mucin secretion through the counter-regulation of histamine receptor by βARK1 (Fig. 9B). This may be relevant in controlling excessive histamine release into the conjunctiva.

It is currently not known if any cells in the conjunctiva, including goblet cells, produce and secrete LXA₄. Along these lines, Gronert et al. have demonstrated that the epithelial cells of the cornea endogenously express LXA₄ and the amount is increased upon wounding23. This LXA₄ could then diffuse via the tears to the goblet cells to stimulate mucin secretion.

Figure 5. LXA₄ Blocks Histamine-stimulated Glycoconjugate Secretion from Rat and Human Goblet Cells. Goblet cells from rat (A) or human (B) were preincubated with LXA₄ (10⁻¹⁰–10⁻⁸ M) for 30 min prior to addition of histamine (His, 10⁻⁵ M) for 2 h and glycoconjugate secretion measured by ELLA. Data are mean ± SD from 3 rats or 3 individuals. *indicates significance difference from histamine alone.
While LXA₄ is an appreciated pro-resolution mediator, the results from several studies indicate that LXA₄ and other pro-resolution mediators can also play a role within other organs in physiological conditions that maybe organ specific. For example, LXA₄ is endogenously produced in the cornea and lacrimal gland under non-inflamed conditions. RvD₁ and AT-RvD₁, similar to LXA₄, alone stimulate conjunctival goblet cell functions. These results imply that these mediators could assist in the maintenance of the normal homeostasis of the ocular surface by regulating goblet cell mucin secretion that is linked to ocular surface health.
Allergic conjunctivitis is the most common type of inflammation of the ocular surface. In this condition, histamine interacts with H1-H4 histamine receptors, all of which are expressed in rat and human conjunctival goblet cells. Histamine also increases \([\text{Ca}^{2+}]_i\) and mucin secretion in a concentration dependent manner. Pre-incubation with LXA4 blocked histamine-stimulated increase in \(\text{[Ca}^{2+}]_i\), mucin secretion and ERK 1/2. Thus, LXA4 likely acts as a pro-resolution mediator acting on goblet cells of the conjunctiva to return mucin levels to normal. LXA4 is likely to have similar effects on histamine-stimulated responses in other tissues. For example, LXA4 inhibits histamine release from human lung mast cells and histamine-stimulated paw edema in mice. This study examined the actions of LXA4 on conjunctival goblet cells only. The ocular surface consists of multiple cell types and is covered by tears, which are a complex film that overspreads the ocular surface. Cultured human goblet cells often react similarly to LXA4 as cultured rat goblet cells. In goblet cells from both species, LXA4 stimulated an increase \(\text{[Ca}^{2+}]_i\) and mucin secretion to the same extent. Mucin secretion stimulated by cysteinyl leukotrienes in human goblet cells was also similar to that obtained with rat goblet cells. There does appear to be several differences between rat and human goblet cells. In human goblet cells, the concentration LXA4 required to maximally inhibit histamine-stimulated increase in \(\text{[Ca}^{2+}]_i\) was 10 fold less than that required in rat goblet cells. An additional difference was demonstrated by experiments involving interactions of LXA4 and RvD1 with their receptors. In rat goblet cells, initial addition of either LXA4 or RvD1 blocked the increase in \(\text{[Ca}^{2+}]_i\), stimulated by a second addition of either LXA4 or RvD1 indicating that these two SPMs bind to the same receptor. However in human goblet cells, while an initial addition of LXA4 blocks the RvD1 response, an initial addition of RvD1 does not block the LXA4 response. In addition, BOC-2 does not alter RvD1-stimulated increase in \(\text{[Ca}^{2+}]_i\). These results support the notion that in human cells RvD1 preferentially activates GPR32 while LXA4 activates both receptors. At this point it is not known if a rat homolog of GPR32 is present and functional in rat goblet cells. There are many other situations in which rat differs from human including regulatory T cell phenotypes, wound healing in skin, and glomerulonephritis. We previously showed the mechanism by which RvD1 prevents the actions of histamine in rat goblet cells. We found that RvD1 counter-regulates the H1 histamine receptor by activation of both \&ARK1 and PKC to prevent the H1 specific agonist-stimulated increase in \(\text{[Ca}^{2+}]_i\). In contrast to RvD1, only an inhibitor of \&ARK1 reversed the LXA4 inhibition of H1 histamine receptor. Cooray et al. have demonstrated that ALX/FPR2 receptor can form hetero- and homodimers depending on the agonist bound. Thus RvD1 and LXA4 could form different dimer formations in rat goblet cells. The signaling pathways activated by LXA4, after binding to ALX/FPR2 are dependent on the cell type. LXA4 acting through ALX/FPR2 stimulated an increase in \(\text{[Ca}^{2+}]_i\), chemotaxis and adherence in human monocytes. In human neutrophils, ALX/FPR2 activation leads to lipid remodeling, arachidonic acid release, and

Figure 7. LXA4 Blocks Histamine-stimulated ERK 1/2 Activation in Rat Goblet Cells. Rat goblet cells were preincubated with LXA4 (10⁻¹⁰⁻¹⁰⁻⁸ M) for 30 min prior to addition of histamine (His, 10⁻⁶ M) for 5 min and amount of activated (phosphorylated) and total ERK determined by Western blot analysis. Representative blot is shown in (A). Upper blot has been rearranged for ease of comparison. Mean ± SD from 4 rats are shown in (B). *indicates significant difference from histamine alone.
activation of phospholipase D (PLD) via PKC with a small increase in \([\text{Ca}^{2+}]_i\). LXA4 had no effect on \([\text{Ca}^{2+}]_i\) in human astrocytoma cells while it increased \([\text{Ca}^{2+}]_i\) in human bronchial epithelia as well as increased the number of tight junctions and Cl\(^-\) secretion and decreased Na\(^+\) absorption\(^{37-39}\). Thus LXA4 can have variable effects on \([\text{Ca}^{2+}]_i\) and other cell functions and cellular responses to LXA4 need to be determined for each cell type.

In conclusion, we demonstrate that ALX/FPR2 receptors are present on cultured human goblet cells, and that LXA4 alone increases \([\text{Ca}^{2+}]_i\), mucin secretion and ERK 1/2 activation. In addition, LXA4 counter-regulates the H1 histamine receptor to block its activation thereby returning the ocular surface to homeostasis. LXA4 thus plays a critical role in ocular surface health and maintenance in physiological conditions. In addition, LXA4 protects the ocular surface from challenges of the external environment that induce ocular surface inflammatory and allergic diseases. Thus LXA4 and this receptor axis may provide the basis for new therapeutic treatments for these diseases.

### Materials and Methods

Synthetic LXA4 was purchased from EMD Millipore (Billerica, MA) and RvD1 was purchased from Cayman Chemical, Ann Arbor, MI. Both compounds were dissolved in ethanol as supplied by the manufacturer and were stored at \(-80^\circ\text{C}\) with minimal exposure to light. Immediately prior to use, the SPMs were diluted in with Krebs-Ringer bicarbonate buffer with HEPES (KRB-HEPES, 119 mM NaCl, 4.8 mM KCl, 1.0 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), 10 mM HEPES, and 5.5 mM glucose [pH 7.45]) to the desired concentrations and added to the cells. The cells were then incubated at 37°C in the dark. Daily working stock
dilutions were discarded following each experiment. N-BOC-Phe-Leu-Phe-Leu-Phe (BOC-2) was purchased from Genescript (Piscataway, NJ).

**Human Tissue.** Human conjunctiva was obtained from Eversight (Ann Arbor, MI). Tissue was placed in Optisol media within 18 h after death.

**Animals.** Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing between 125 and 150 g were anesthetized with CO2 for 1 min, decapitated, and the bulbar and fornical conjunctival membranes removed from both eyes. All experiments were approved by the Schepens Eye Research Institute Animal Care and Use Committee and carried out in accordance to the protocols approved by this committee.
Human monocytes; THP1 | [Ca²⁺], chemotaxis, adherence | 33,34

Human neutrophils | [PKC to PLD; AA release; small increase in [Ca²⁺]; chemotaxis; apoptosis delay] | 24,35,46

Human astrocytoma cells | IL-1β induced IL-8 and ICAM-1; no effect on [Ca²⁺], | 36

Human bronchial epithelia | Tight junctions, [Ca²⁺], CI secretion and ALX translocation, air/surface height; ENaC activity | 38

**Table 1. LXA₄ via ALX/FPR2 Signaling in Human Cell Types.** PKC- protein kinase C; PLD – phospholipase D; AA – arachidonic acid; IL-1β - interleukin 1β; IL-8 – interleukin 8; ICAM-1 - intercellular adhesion molecules; EnaC- epithelial sodium channel.

**Cell Culture.** Goblet cells from human and rat conjunctiva were grown in organ culture as described and extensively characterized previously[^6][^17][^40][^42]. The tissue plug was removed after nodules of cells were observed. First passage goblet cells were used in all experiments. The identity of cultured cells was periodically checked by evaluating staining with antibody to cytokeratin 7 (detects goblet cell bodies) and the lectin Ulex europeus agglutinin (UEA-I) (detects goblet cell secretory product) to ensure that goblet cells predominated.

**Measurement of [Ca²⁺]i.** Goblet cells were incubated for 1 h at 37 °C with KRB-HEPES with 0.5% BSA containing 0.5 μM fura-2/AM (Invitrogen, Grand Island, NY), 8 μM pluronnic acid F127, and 250 μM sulfipyrazone followed by washing in KRB-HEPES containing sulfipyrazone. Inhibitors were added for the last 30 min of the fura-2 incubation. Calcium measurements were made with a ratio imaging system (InCyt Im2; Intracellular Imaging, Cincinnati, OH) using wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. At least 10 cells were selected in each experimental condition. Data were collected in real time and are presented as the average of the basal value (no added agonist) from the peak [Ca²⁺]i.

**Measurement of Glycoconjugate Secretion.** Cultured goblet cells were serum starved for 2 h before use and then stimulated with either LXA₄ or histamine in serum-free RPMI 1640 supplemented with 0.5% BSA for 2 h. Inhibitors were added 30 min prior to stimulation. Goblet cell secretion was measured using an enzyme-linked lectin assay (ELLA) with the lectin UEA-I. UEA-1 detects high molecular weight glycoconjugates containing 1-fucose including mucin MUC5AC produced by goblet cells. The media were collected and analyzed for the amount of lectin-detectable glycoconjugates, which quantifies the amount of goblet cell secretion as described earlier[^27]. Glycoconjugate secretion was expressed as fold increase over basal that was set to 1.

**Reverse Transcriptase (RT)-PCR.** Cultured human goblet cells were homogenized in TRIzol and total RNA was isolated. One microgram of purified total RNA was used for complementary DNA (cDNA) synthesis using the Superscript First-Strand Synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). The cDNA was amplified by the polymerase chain reaction (PCR) using primers specific to human ALX/FPR2 receptor using the Jumpstart REDTaq ReadyMix Reaction Mix (Sigma-Aldrich, St. Louis, MO) in a thermal cycler (Master Cycler, Eppendorf, Hauppauge, NY). The primers were from published sequences[^44]. The forward primer sequence was GGA TTT GCA CCC ACT GCA TTT and reverse primer was ATC CAA GGT CCG AGA TCA C. These primers generated a product of 528 base pairs.

**Western blotting analyses.** Cultured goblet cells were homogenized in RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The lysate was centrifuged at 2000 g for 30 min at 4 °C. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and processed for western blotting. The antibody against the ALX/FPR2 receptor (Novus Biologics, Littleton, CO) was diluted 1:1000. To measure activation of ERK 1/2, LXA₄ was added 30 min prior to histamine (10⁻⁷ M) for 5 min. The antibody against phosphorylated (active) ERK 1/2 was used diluted 1:200 and total ERK 1/2 (Santa Cruz Biotechnologies, Santa Cruz, CA) was diluted 1:500. Immunoreactive bands were visualized by the enhanced chemiluminescence method. The films were analyzed with Image J software (http://rsweb.nih.gov/ij/). Values for phosphorylated ERK 1/2 were normalized to total ERK 1/2. Control value was set as 1.

**Immunofluorescence Microscopy.** First passage cells were grown on glass cover slips and were fixed in 4% formaldehyde diluted in phosphate buffered saline (PBS, 145 mM NaCl, 7.3 mM Na₂HPO₄, and 2.7 mM NaH₂PO₄...
(pH 7.2)) for 4 hours at 4°C. The coverslips were rinsed for 5 minutes in PBS, and nonspecific sites were blocked by incubation with 1% bovine serum albumin, and 0.2% Triton X-100 in PBS for 45 minutes at room temperature. ALX/FPR2 receptor antibody (Novus Biologics) was used at 1:100 dilution overnight at 4°C. EA1-1 directly conjugated to FITC (Sigma-Aldrich, St. Louis, MO) was used at a dilution of 1:300 to identify goblet cells. Secondary antibodies were conjugated to Cy 3 (Jackson ImmunoResearch Laboratories, West Grove, PA) was used at a dilution of 1:150 for 1 h at room temperature. Negative control experiments included incubation with the isotype control antibody. The cells were viewed by fluorescence microscopy (Eclipse E80i; Nikon, Tokyo, Japan) and micrographs were taken with a digital camera (Spot; Diagnostic Instruments, Inc, Sterling Heights, MI).

**Statistical analysis.** Results were expressed as the fold-increase above basal. Results are presented as mean ± SD. Data were analyzed by ANOVA followed by post-hoc Tukey or Student's t-test. P < 0.05 was considered statistically significant.

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Author Contributions

R.R.H. conducted experiments, designed experiments, acquired data, analyzed data, wrote manuscript. D.L. conducted experiments. M.A.S. conducted experiments. C.N.S. provided reagents, designed experiments, wrote manuscript. D.A.D. designed experiments, wrote manuscript.

Additional Information

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