Aspirin-triggered lipoxin enhances macrophage phagocytosis of bacteria while inhibiting inflammatory cytokine production

David Prescott and Derek M. McKay
Gastrointestinal Research Group, Department of Physiology and Pharmacology, Calvin, Phoebe and Joan Snyder Institute of Infection, Immunity and Inflammation, University of Calgary, Calgary, Alberta, Canada

Submitted 3 February 2011; accepted in final form 6 June 2011

Prescott D, McKay DM. Aspirin-triggered lipoxin enhances macrophage phagocytosis of bacteria while inhibiting inflammatory cytokine production. Am J Physiol Gastrointest Liver Physiol 301: G487–G497, 2011. First published June 9, 2011; doi:10.1152/ajpgi.00042.2011.—The macrophage plays a major role in the induction and resolution phases of inflammation; however, how lipid mediator-derived signals may modulate macrophage function in the resolution of inflammation driven by microbes (e.g., in inflammatory bowel disease) is not well understood. We examined the effects of aspirin-triggered lipoxin (ATL), a stable analog of lipoxin A4, on the antimicrobial responses of human peripheral blood mononuclear cell-derived macrophages and the monocytic THP-1 cell line. Additionally, we assessed the expression and localization of the lipoxin receptor, formyl peptide receptor 2 (FPR2), in colonic mucosal biopsies from patients with Crohn’s disease to determine whether the capacity for lipoxin signaling is altered in inflammatory bowel disease. We found that THP-1 cells treated with ATL (100 nM) displayed increased phagocytosis of inert fluorescent beads and Escherichia coli in a scavenger receptor- and PI3K-dependent, opsonization-independent manner. This ATL-induced increase in phagocytosis was also observed in primary human macrophages, where it was associated with an inhibition of E. coli-induced IL-1β and IL-8 production. Finally, we found that FPR2 gene expression was increased approximately sixfold in the colon of patients with Crohn’s disease, a finding reproduced in vitro by the treatment of THP-1 cells with interferon-γ or lipopolysaccharide. These results suggest that lipoxin signaling is upregulated in inflammatory environments, and, in addition to their known role in tissue resolution following injury, lipoxins can enhance macrophage clearance of invading microbes.

INFLAMMATION WAS ONCE VIEWED as a process that simply dissipated after running its course. However, it is now clear that the resolution phase of inflammation is not passive but rather requires a distinct set of signals to shut off the inflammatory machinery and return tissues to a homeostatic state (52). One such signal is lipoxin A4 (LXA4), an eicosanoid derived from the metabolism of arachidonic acid by the sequential action of two lipoxygenases (LO), or by aspirin-acetylated cyclooxygenase (6). LXA4 is a major ligand of the formyl peptide receptor (FPR2), otherwise known as ALX (9). Administration of exogenous lipoxin or stable analogs of lipoxin has been shown to be protective in murine models of ischemia-reperfusion (59), peritonitis (1), and experimental asthma (33). Similarly, mice deficient in FPR2 or 15-LO, an enzyme required for the synthesis of lipoxins, are more susceptible to experimental inflammation (9, 29). LXA4 exerts a number of anti-inflammatory effects, such as the inhibition of neutrophil recruitment to inflamed tissue (43) and the enhancement of macrophage phagocytosis of apoptotic neutrophils within an inflamed tissue (16). Thus lipoxins and the promotion of the resolution of inflammation have emerged as potential targets to treat or cure chronic inflammatory disorders.

The inflammatory bowel diseases (IBD), Crohn’s disease (CD) and ulcerative colitis (UC), are lifelong, debilitating conditions characterized by severe, intermittent inflammation of the gastrointestinal tract (58). A cure for IBD remains elusive, since the etiology of IBD has yet to be determined, although evidence from murine models of colitis (36) and analyses of patients (3) suggest that the inflammation is the result of an altered immune response to the commensal microflora. Therapeutic agents that promote the resolution of inflammation have been suggested as a new approach to treat intestinal inflammation, since disease can be alleviated in murine models of experimental colitis by the administration of LXA4 analogs (11, 14). Despite this, the kinetics and dynamics of the resolution of microbe-driven inflammation remain poorly understood.

An important member of the inflammatory milieu in IBD is the macrophage. These cells play critical roles in mucosal maintenance and defense, being responsible for internalizing and degrading dead cells, debris, and pathogens and their products and for presenting protein antigens to T cells. Macrophages can be activated by a variety of cytokines and bacteria-derived signals to release both pro- and anti-inflammatory mediators (42). Under healthy conditions, macrophages residing in the intestinal mucosa are relatively quiescent, producing only small quantities of cytokines and proteins involved in antigen presentation when challenged with stimuli such as bacteria (53). However, circulating monocytes are heavily recruited to inflamed regions of the colon in patients with IBD (28), where they differentiate and are a major source of proinflammatory cytokines (e.g., TNF-α) (21), suggesting that newly recruited macrophages play a role in the immunopathology of IBD.

Given the importance of macrophages in the surveillance of the intestinal microbiota and the resolution of inflammatory processes, we chose to examine the effects of lipoxin signaling on the response of macrophages to a laboratory commensal strain of Escherichia coli. Initial investigation revealed an upregulation of the lipoxin receptor on macrophages in biopsies from patients with CD. Furthermore, peripheral blood mononuclear cell-derived macrophages treated with aspirin-triggered lipoxin (ATL) showed increased bacterial phagocytosis and decreased production of inflammatory cytokines. Taken together, these findings indicate that lipoxin-driven macrophage activity may play a critical role in the maintenance
of an aseptic environment following injury or inflammation in areas of heavy microbial load, such as the gut.

METHODS

Reagents

Reagents and inhibitors were purchased from: ATL (VWR International, Edmonton, AB, Canada); S. cerevisiae mannin, lipopolysaccharide (LPS), and F-Met-Leu-Phe (Sigma-Aldrich Canada, Oakville, ON, Canada); AS605240, WRW4, and AC2-26 (Cedarlane Laboratories, Burlington, ON, Canada); recombinant human IFN-γ, TNF-α, IL-4, and IL-13 (Ebioscience, San Diego, CA).

Human Samples

To determine whether the expression of various genes involved in the production and signaling of lipoxin was altered in human IBD, colonic pinch mucosal biopsies were obtained with ethical approval from the University of Calgary Intestinal Inflammation Tissue Bank. Patient groups consisted of healthy controls undergoing screening for colon cancer (n = 7), patients with CD in remission (n = 8), and patients with active CD, with biopsies taken from both inflamed and noninflamed regions of the colon (n = 10). Patient details are reported in Table 1.

Cell Culture

THP-1 monocytes.

The human monocytic THP-1 cell line (ATCC, Manassas, VA) was maintained in RPMI-1640 medium containing 2% penicillin-streptomycin and 10% fetal bovine serum, and added to 12-well cell culture plates (Becton, Dickinson, Mississauga, ON, Canada) at a density of 2.5 × 10^5 cells/well. Differentiation into an adherent macrophage-like phenotype was achieved by incubation with 10 nM PMA (Sigma) for 24 h. Following washes with fresh medium, cells were left for 24 h prior to experimentation (12).

Human PBMCs.

Human peripheral blood mononuclear cells (PBMCs) were extracted from whole blood samples from healthy volunteers with ethical approval. Briefly, whole blood preparations were diluted twofold in 37°C PBS, and Ficoll-Paque PLUS (GE Healthcare Canada, Baie d’Urfe, QC, Canada) was gently layered beneath the blood, followed by a 30-min centrifugation at 300 g (without brakes). The buffy coat containing the PBMCs was collected and washed twice in 37°C PBS, and the cells were resuspended in serum-free THP-1 medium, then plated at a density of 2.5 × 10^5/well. After a 2-h incubation at 37°C, nonadherent cells were removed and the adherent macrophages were washed three times with 37°C PBS. PBMCs were maintained in complete RPMI medium and cultured for 5–7 days to ensure the development of a macrophage-like phenotype.

Cytokine Treatment

Differentiated THP-1 and isolated macrophages were treated with 10 ng/ml of IFN-γ, TNF-α, IL-4, or IL-13, or 1 µg/ml LPS for 6 or 24 h before being collected for mRNA expression analysis.

Fluorescence Microscopy

Protein expression and localization were visualized by immunostaining. Formalin-fixed biopsies were dehydrated by sequential graded alcohol washes and embedded in Paraplast (VWR), and 5-µm sections were collected on poly-d-lysine-coated slides. Sections were rehydrated and antigen-retrieval performed by incubating slides in antigen-retrieval solution (TRIS-EDTA, 1% Triton X-100, pH 9.0) in a vegetable steamer for 30 min. Tissue sections were blocked for 1 h with immunofluorescence buffer (TBS, 1% Triton X-100, 1% bovine serum albumin, 2% normal rabbit serum) before overnight incubation at 4°C with rabbit-anti FPR2 (1:100, Abcam, Cambridge, MA) or mouse-anti CD68 (1:100, Dako Canada, Mississauga, ON, Canada). After extensive washes, sections were treated with 2 µg/ml of the appropriate fluorochrome-labeled secondary antibodies (Invitrogen Canada, Burlington, ON, Canada) for 1 h at room temperature. Fluorescence was visualized on an argon-laser-powered fluorescent microscope (Olympus Canada, Markham, ON, Canada) fitted with NIB (excitation 470–490 nm; emission 505 nm; Olympus) and WIG (excitation 520–550 nm; emission >580 nm; Olympus) band-pass filter cubes. Images were recorded by a microscope-mounted digital camera and processed by using the QCapture Pro imaging software (QImaging, Surrey, BC, Canada). Target expression was quantified by counting the average number of immunoreactive cells in three high-power fields.

mRNA Analysis

RNA was extracted from cell culture samples with the Trizol (Invitrogen) phenol/chloroform method as per the manufacturer’s instructions and from human samples by use of PureLink RNA extraction spin columns (Invitrogen). Extracted RNA (100–500 ng) was reverse transcribed by using the iScript RT kit (Bio-Rad Laboratories Canada, Mississauga, ON, Canada) in a MyCycler thermocycler (Bio-Rad). One microliter of cDNA was added to a reaction buffer containing 300 nM gene-specific primers (see Table 2 for primer sequences) and reaction mix containing platinum Taq polymerase (Invitrogen). The following parameters were used: initial denaturing (94°C for 5 min), 35 cycles (denaturing at 94°C for 30 s, annealing at 58°C for 5 s, extension at 72°C for 10 s), and a final extension at 72°C for 5 min. The PCR products were then electrophoresed through 2% agarose gels containing 0.5 mg/ml ethidium bromide and visualized under UV light. Alternatively, 1 µl of cDNA was added to a reaction buffer containing 1 × SYBR green reaction mix (Bio-Rad) and 300 nM gene-specific primers (Table 2). Primers were validated for efficiency by performing serial log dilutions of a single sample and examining the slope of the resulting standard curve. Primer efficiencies of <0.9 were considered acceptable. Quantitative PCR reaction was performed in a Mastercycler Real Time PCR Thermocycler (Eppendorf Canada, Mississauga, ON, Canada), with the following program: 1 s initial denaturation (95°), followed by 45 cycles (1 s denaturing, 95°C; 30 s annealing and extension, 55°C). Data were analyzed by use of the Realplex software platform (Eppendorf). Data were expressed by the 2^−ΔΔCT method with 18s RNA used as a housekeeping gene, and

Table 1. Human tissue sample donor characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Age, mean ± SE</th>
<th>Male-to-Female Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Control</td>
<td>45.0 ± 15.0</td>
<td>4/3</td>
</tr>
<tr>
<td>Inactive Disease</td>
<td>45.3 ± 18.4</td>
<td>5/3</td>
</tr>
<tr>
<td>Active Disease</td>
<td>42.8 ± 11.3</td>
<td>5/5</td>
</tr>
</tbody>
</table>

Table 2. Nucleotide sequences of PCR primers use in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPR2</td>
<td>GAACCCATGCTTTAGCTCT</td>
<td>ATATCCGGACCATCCATCTCT</td>
</tr>
<tr>
<td>5-LO</td>
<td>ACTGGAGACAGCGCCAGAACAGT</td>
<td>TTCCCTGAAAGTCGGCCAGAAT</td>
</tr>
<tr>
<td>15-LO-1</td>
<td>GAACATCTCAGAGAGCTTCGC</td>
<td>ATGGTCAAGGACAGCATGTGTTTCCC</td>
</tr>
<tr>
<td>15-LO-2</td>
<td>GACCTATCCGAGTACACTTTTGGGTG</td>
<td>TTCCCTTGGGGTACCATCTATTC</td>
</tr>
<tr>
<td>18 s rRNA</td>
<td>ATGCTGGTCTTGGTTGGTGGAG</td>
<td>CGTATGCCGACACTCAAGAGT</td>
</tr>
<tr>
<td>CD86</td>
<td>AGAGATTACCTACGAGGAGG</td>
<td>ATCCACACATGACAGCAAGT</td>
</tr>
<tr>
<td>MMR</td>
<td>GGCGGTCGACCTCAGAAGTAT</td>
<td>AGCAAGCGATTTGCTGAAGACG</td>
</tr>
<tr>
<td>iNOS</td>
<td>AGACAGATCAAGTATCCACGAGG</td>
<td>AATCCATGCTTGGTTGAGGACAGAAGT</td>
</tr>
<tr>
<td>CD14</td>
<td>GGGCGTTGTTGAAGAAGAAG</td>
<td>GTTGAAGTGTGTGAGAAGATG</td>
</tr>
<tr>
<td>CD16a</td>
<td>TTTTTCATTGGCAGACATCCG</td>
<td>TTCAAGTGCTTTTGAGGATG</td>
</tr>
<tr>
<td>CCR7</td>
<td>GATGAGGAGCTTCCTGACATG</td>
<td>TAGAGGAGGAGCTTGAGAAGT</td>
</tr>
<tr>
<td>CXCR4</td>
<td>GGGTGTGAGTATGTTGGCTG</td>
<td>TGGAGTGTGAGGACATGAGG</td>
</tr>
</tbody>
</table>

FPR2, formyl peptide receptor 2; LO, lipoprotein; MMR, mannose receptor; iNOS, inducible nitric oxide synthase.

Downloaded from http://ajpgi.physiology.org/ on October 14, 2017.
normalized to naive cell (cell culture) or healthy control (human biopsy) expression levels (49).

**Western Blot**

Protein was extracted from cells and tissues by freezing in modified RIPA buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. For phosphorylation studies, RIPA buffer contained 1 mM Na3VO4 to inhibit phosphatase activity. Protein concentrations were assessed with a modified Bradford reagent assay (Bio-Rad). Twenty micrograms of protein were boiled for 5 min in Laemmli buffer and subjected to discontinuous SDS-PAGE (4% stacking pH 6.8, 10% separating pH 8.8). Proteins were then transferred to polyvinylidene difluoride membranes and blocked for 1 h at 20°C in 5% skim milk-TBST (Tris-buffered saline, 0.1% Tween 20). Membranes were incubated with a rabbit monoclonal antibody to FPR2 (1:6,000 in 5% skim milk-TBST, 1 h, Abcam) or phosphorylated and total Akt (1:1,000 in 5% BSA/TBST, Cell Signaling Technology, Danvers, MA) followed by a horseradish peroxidase-conjugated secondary antibody (1:20,000 for FPR2, 1:10,000 for others, 1 h, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed 3 × 5 min in 5% milk-TBST between each step. Membranes were then exposed to Western Lightning Plus enhanced chemiluminescence solution (ECL, PerkinElmer, Woodbridge, ON, Canada) for 30 s, exposed to X-Omat Blue film (PerkinElmer) for 30 s to 5 min and developed on an automated film developer machine (Eastman Kodak Canada, Toronto, ON, Canada).

**Bacterial Internalization**

Bacterial internalization was assessed by the gentamicin exclusion assay. Briefly, THP-1 macrophages were exposed to 1 × 10⁶ colony-forming units (CFU) of *E. coli* strain HB101 for 2–24 h. Macrophages were then washed with PBS and exposed to 200 µg/ml gentamicin (Invitrogen) in THP-1 medium for 2 h. Cells were then washed with PBS and lysed with 1% Triton X-100 (Sigma) in cold PBS for 20 min. Lysates were serially diluted and 10 µl/dilution plated on LB agar (EMD Chemicals, Gibbstown, NJ) in triplicate to assess numbers of viable bacteria. Opsonization was achieved by incubation with *E. coli* opsonizing reagent (Rabbit Polyclonal antibody against *E. coli*, 0.001 units, Invitrogen) for 1 h at 37°C.

**Fluorescent Bead Internalization**

Internalization of inert particles (Invitrogen) was assayed through the use of 1 µm carboxylate-modified microspheres loaded with fluorescent dye. Each macrophage preparation was exposed to 5 × 10⁶ microspheres and allowed to incubate for 2–24 h. Following five washes with PBS, microtiter plates were read on a Wallac Victor2 multilabel counter (PerkinElmer), with excitation and emission filters of 580 and 605 nm, respectively. Wells without beads were assessed to determine background fluorescence. Data are expressed as a % change in fluorescence in treated vs. control wells. Internalization was also visualized by fluorescent microscopy. THP-1 monocytes were seeded on Lab-Tek chamber slides (Nunc, Rochester, NY) at a density of 5 × 10⁴ cells/chamber and differentiated and maintained as above. Cells were exposed to 5 × 10⁶ microspheres for 6 h and washed five times with PBS. Cells were then fixed for 10 min in 100% methanol at −20°C and rinsed in PBS, and nuclei were counterstained by incubation with 0.5 µg/ml 4,6-diamidino-2-phenylindole (Invitrogen) for 5 min. Slides were then mounted in Vectashield aqueous mounting medium (Vector Laboratories, Burlington, ON, Canada) and fitted with a coverslip (VWR). Fluorescence was visualized on an argon-laser-powered fluorescent microscope (Olympus Canada, Markham, ON, Canada) fitted with a WIG filter cube (excitation 520 to 550 nm; emission >580 nm; Olympus), and cell morphology was visualized by phase-contrast microscopy. Images were recorded by a microscope-mounted digital camera and processed by use of the QCapture Pro imaging software (QImaging, Surrey, BC, Canada). Fluorescent images were quantified for internalization by counting the total number of internalized beads per cell in three low-power fields.

**Cytokine Release Assay**

PBMC-derived macrophages and differentiated THP-1 cells were incubated with 10⁶ CFU of live *E. coli* HB101 for 6 or 24 h. Supernatants were collected and centrifuged at 12,500 g for 10 min to remove bacteria and levels of IL-1β, TNF-α, IL-8, and IL-10 determined by ELISA following the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

**Statistical Analysis**

Data were analyzed in the Prism 5 software (GraphPad Software, La Jolla, CA) by one-way ANOVA followed by Tukey’s post hoc test or paired Student’s t-test (Figs. 1D, 5, and 7C). Statistical significance was judged to be at *P* < 0.05. Data are presented as mean values ± SE, and *n* refers to the number of patients, PBMC preparations, or THP-1 culture wells used in the experiment.

**RESULTS**

**ATL Amplifies the Phagocytic Activity of Primary and THP-1 Macrophages**

To determine whether lipoxin alters macrophage phagocytosis, THP-1 cells were exposed to fluorescent polystyrene beads. With a 2-h ATL (100 nM) treatment, there was a 16 ± 8% (*P* < 0.05) increase in THP-1 phagocytosis of beads. Figure 1A shows the dose dependency of the ATL-enhancement of phagocytosis of inert beads 6 h posttreatment. This increased phagocytosis as shown by total fluorescence was supported by imaging that revealed significantly more beads per cell in ATL-treated THP-1 cells (Fig. 1B). Similarly, 6-h treatment with 100 nM ATL resulted in a significant increase in the phagocytosis of *E. coli*, and this effect only occurred when the ATL and bacteria were added simultaneously (Fig. 1C). However, there was no observable difference between the groups after 24 h of exposure to *E. coli* ± ATL (data not shown), suggesting an immediate effect of the lipoxin and that the limiting conditions in vitro allow the nontreated macrophages to eventually catch up with the lipoxin-treated cells. This effect was also replicated in macrophages derived from PBMCs isolated from healthy control subjects (Fig. 1D), since treatment of adherent blood monocytes with 100 nM ATL resulted in a 32.2 ± 15.8% increase in phagocytosis of live bacteria compared with controls.

**The Prophagocytic Effect on Macrophages Is Stimulated by Lipid but not Protein Agonists of the FPR2 Receptor**

Because FPR2 is a promiscuous receptor with high similarity to FPR1, we wanted to determine the receptor selectivity of the enhanced phagocytosis. ATL, but neither the FPR1 agonist N-formyl-methionine-leucine-phenylalanine nor the peptide FPR2 agonist AC2-26, significantly increased the phagocytosis of inert beads (Fig. 2A) or live *E. coli* (Fig. 2B), indicating that this effect is specific to lipid agonists of the FPR2 receptor. Competitive inhibition of FPR2 by AC2-26 (1 µM) or the FPR2-selective peptide inhibitor WRW4 (200 nM) failed to inhibit the increased phagocytosis in response to ATL (data not shown), indicating that the increased phagocytosis elicited by ATL is dependent on the lipid-binding moiety of FPR2. Although these observation sup-
port ATL signaling through FPR2, definitive proof of this will require molecular knockdown or knockout of the receptor.

**ATL-Induced Phagocytosis Is Dependent on the Mannose Scavenger Receptor**

Treatment of THP-1 cells with 1 mM mannose completely blocked the ATL-enhanced phagocytosis of *E. coli* (Fig. 3). In contrast, although opsonization (coating with IgG to allow Fc receptor-mediated phagocytosis) of the *E. coli* resulted in increased internalization ($5.85 \pm 3.65$ vs. $0.33 \pm 0.07\%$ internalization, $n = 4$), this was not further enhanced by treatment with ATL ($3.52 \pm 3.41\%$ internalization, $n = 4$), suggesting that lipoxins induce an increase in phagocytosis in an opsonization-independent manner.

---

**Fig. 1.** Aspirin-triggered lipoxin (ATL) upregulates phagocytosis by THP-1 macrophages. One-micrometer fluorescent beads are more readily phagocytosed by macrophages exposed to ATL (6 h), in a dose-dependent manner (A, mean ± SE, $n = 6$, *P < 0.05 and **P < 0.01 compared with naive controls). This phenomenon was visualized by fluorescence microscopy and quantified by counting the number of internalized fluorescent beads per cell in 3 low-power fields (B, mean ± SE, $n = 3$ ***P < 0.001 compared with naive controls). ATL also amplifies the phagocytosis of live nonpathogenic *Escherichia coli* (strain HB101), but only if ATL and bacteria are added simultaneously (C, $n = 6$, **P < 0.01, significantly different from other groups.) This effect was also observed in monocytes/macrophages from healthy volunteers (D, $n = 6$ subjects, paired values, **P < 0.01 compared with *E. coli* only; horizontal bar indicates the mean).
ATL-Induced Phagocytosis Occurs in a Phosphoinositol 3-Kinase p110γ-Dependent Manner

Because lipoxins are known to signal through a phosphoinositide 3-kinase (PI3K)-mediated pathway in macrophages (44), and FPR2 is a G protein-coupled receptor (9), we chose to examine whether the GPCR-associated PI3K isoform PI3K p110γ (54) plays a role in ATL-mediated induction of phagocytosis. The amplification of E. coli phagocytosis by ATL was inhibited by treatment with the PI3K p110γ-selective inhibitor AS605240 (10 nM, Fig. 4A). Moreover, Western blot analysis reveals an increase in phosphorylation of the PI3K downstream effector molecule Akt following treatment with ATL for 5 min, and this was blocked by 30-min pretreatment with AS605240 (10 nM, Fig. 4B). These effects were not the result of AS605240-induced cytotoxicity, since THP-1 macrophages exposed to 100 nM AS605240 in the presence of E. coli for 6 h displayed greater than 90% viability as measured by the Trypan blue exclusion test.

The Increase in Phagocytosis Induced by ATL Is Associated with a Decrease in IL-1β and IL-8 from PBMC-Derived Macrophages but not THP-1 Cells

Lipoxins are known to inhibit proinflammatory cytokine release in response to many stimuli (8, 57). Accordingly, we found that 24-h treatment with ATL led to a significant decrease in the production of both IL-8 (32.3 ± 7.5% decrease compared with E. coli alone) and IL-1β (16.6 ± 12.0% decrease compared with E. coli alone; n = 6) in response to E.
coli, despite an associated increase in bacterial internalization (Fig. 5, A and B). No significant differences in TNF-α or IL-10 production were observed (Fig. 5, C and D). This phenomenon was not replicable in THP-1 macrophages, since treatment with ATL did not significantly affect E. coli-induced IL-8 production, and surprisingly it induced a small increase in IL-1β production compared with E. coli alone (Table 3). Naive macrophages and macrophages treated with ATL alone produced negligible levels of all cytokines assessed in both the primary cells and the cell line.

**Treatment with ATL Does not Diminish Macrophage Antimicrobial Function and Upregulates the Chemokine Receptor CCR7**

ATL-treated macrophages were able to eliminate all internalized bacteria by 24 h after treatment, suggesting that the increased number of bacteria found within ATL-treated macrophages is not the result of an inability of the macrophage to kill phagocytosed bacteria, but a true increase in early phagocytosis. Additionally, we assessed the expression of genes responsible for the handling of bacteria (CD14, iNOS, MMR), antigen presentation (CD80, CD86), and chemotaxis (CCR7, CXCR4). We found a significant upregulation in the expression of CCR7 upon 2 h of treatment with 100 nM ATL and 10⁶ CFU of E. coli compared with naive cells or cells treated with ATL or E. coli alone (Table 4). None of the other genes tested were significantly affected.

**Table 3. Cytokine production by THP-1 macrophages in response to live Escherichia coli**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>E. coli</th>
<th>E. coli + ATL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β, ng/ml</td>
<td>0.96 ± 0.34</td>
<td>1.41 ± 0.49*</td>
</tr>
<tr>
<td>TNF-α, ng/ml</td>
<td>7.46 ± 0.83</td>
<td>6.40 ± 1.17</td>
</tr>
<tr>
<td>IL-8, ng/ml</td>
<td>7.76 ± 1.84</td>
<td>6.08 ± 0.90</td>
</tr>
<tr>
<td>IL-10, ng/ml</td>
<td>8.89 ± 2.25</td>
<td>7.24 ± 0.44</td>
</tr>
</tbody>
</table>

Data are means ± SD, n = 6. *P < 0.05 compared to E. coli alone; E. coli at 10⁶ cfu inoculum, aspirin-triggered lipoxin (ATL) at 100 nM; cytokines measured 24 h post-E. coli addition.

**FPR2 Expression Is Increased in Inflamed Tissue from Patients with CD**

The analysis of FPR2 mRNA expression in colonic mucosal pinch biopsies by quantitative PCR revealed a sixfold increase in expression in patients with active CD compared with healthy controls, but only in biopsies taken from inflamed areas (Fig. 6A). Patients with CD exhibited no observable differences in colonic gene expression of the lipoxin synthesizing enzymes 5-LO, 15-LO-1, or 15-LO-2. FPR2-like immunoreactivity was localized to both the epithelium and lamina propria in the colon, whereas staining for the macrophage marker CD68 revealed significant colocalization of FPR2 and CD68 in the lamina propria only (Fig. 6B), suggesting that increased macrophage expression of FPR2 is responsible for at least a portion of the increase in FPR2 expression observed in inflamed tissues. This was quantified by assessing the number of immunoreactive double-positive cells under three high-powered fields in biopsies taken from inflamed and noninflamed regions of three separate patients with CD (Fig. 6C).

**THP-1 FPR2 Expression Is Upregulated by Proinflammatory Stimuli**

THP-1 cells constitutively express FPR2 mRNA and protein (Fig. 7A), and both are upregulated following differentiation into

**Table 4. Fold changes in THP-1 macrophage gene expression induced by ATL and E. coli**

<table>
<thead>
<tr>
<th>Gene</th>
<th>ATL</th>
<th>E. coli</th>
<th>E. coli + ATL</th>
<th>ANOVA Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>0.88 ± 0.10</td>
<td>0.77 ± 0.13</td>
<td>1.24 ± 0.36</td>
<td>NS</td>
</tr>
<tr>
<td>MMR</td>
<td>1.35 ± 0.24</td>
<td>1.58 ± 0.35</td>
<td>1.24 ± 0.29</td>
<td>NS</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.58 ± 0.34</td>
<td>1.75 ± 0.45</td>
<td>2.35 ± 0.47</td>
<td>NS</td>
</tr>
<tr>
<td>CD80</td>
<td>1.60 ± 0.41</td>
<td>4.24 ± 1.96</td>
<td>6.45 ± 2.77</td>
<td>NS</td>
</tr>
<tr>
<td>CD86</td>
<td>1.58 ± 0.33</td>
<td>1.58 ± 0.31</td>
<td>2.52 ± 0.62</td>
<td>NS</td>
</tr>
<tr>
<td>CXCR4</td>
<td>1.60 ± 0.46</td>
<td>1.69 ± 0.28</td>
<td>2.25 ± 0.40</td>
<td>NS</td>
</tr>
<tr>
<td>CCR7</td>
<td>1.40 ± 0.37</td>
<td>1.29 ± 0.23</td>
<td>3.00 ± 0.79*</td>
<td>P = 0.0004</td>
</tr>
</tbody>
</table>

Data are means ± SE fold change in gene expression relative to naive THP-1 macrophages; n = 9; *P < 0.05 compared to other groups; E. coli added at 10⁶ cfu/well; ATL = 100 nM; mRNA assessed 24 h posttreatment.
an adherent macrophage-like phenotype following treatment with PMA. Treatment with IFN-γ (10 ng/ml) or LPS (1 μg/ml) for 24 h, but not TNF-α, IL-4, or IL-13, significantly increased THP-1 expression of FPR2 mRNA (Fig. 7B). Similarly, FPR2 mRNA expression in human blood-derived macrophages was significantly increased after exposure to IFN-γ or LPS (Fig. 7C).

**DISCUSSION**

Macrophages respond to microbes, cytokines, and danger signals by releasing a wide variety of proinflammatory mediators (55) and are therefore often thought to play a major etiological role in chronic inflammatory diseases such as IBD.
Indeed, increased recruitment of CD14+ monocytes to the intestinal lamina propria can be a hallmark of CD (18), and the activation of these cells has been implicated in the pathogenesis of murine colitis and human IBD (25, 26). However, macrophages can also play a regulatory role in colitis; multiple studies have shown that genetic ablation of monocytes significantly worsens the outcome of disease (15, 45). This dichotomy in macrophage function can be explained in a number of ways. Macrophage subsets have been described that function in tissue recovery and restitution (38). These macrophages, designated as M2a or alternatively activated macrophages, may have anti-inflammatory activity in the gut (23). Moreover, macrophages are known to play a major role in the eicosanoid-directed resolution of inflammation; LXA4, a well-characterized proresolving molecule, can induce the recruitment of monocytes to inflamed tissues, where it stimulates the clearance of the inflammatory cell infiltrate and helps return the tissue to homeostasis (52).

Although the kinetics and dynamics of lipoxin-driven resolution of inflammation in sterile compartments such as the dorsal air pouch (32) have been characterized, much less is known about the role of lipoxins in the resolution of inflammation that is driven by live bacteria. Because the inflammation seen in IBD is thought to be evoked primarily by the commensal microflora (48), it is of great importance to determine how the presence of microbes affects lipoxin-mediated resolution of inflammation. Consequently the present study focused on the effect of lipoxins on the macrophage’s phagocytic activity and response to bacteria. We chose to use ATL, a lipoxin generated by interactions between 5-LO and aspirin-acetylated cyclooxygenase II (7), because of the high degree of similarity in biological activity between LXA4, B4, and ATL (51), along with an improved half-life.

Treatment with ATL significantly increased the phagocytosis of inert particles and viable E. coli by THP-1 cells (a model of human macrophages) and by PBMC-derived primary macrophages, a biological activity of lipoxin that has not been previously reported. Subsequent mechanistic studies were performed with THP-1 cells, and it is important to note that the increased phagocytosis was dependent on simultaneous exposure to ATL and the E. coli (pretreatment with ATL did not recapitulate this event), suggesting that prophylactic use of lipoxin analogs may be less effective than therapeutic administration. Lipoxins have been shown to induce the phagocytosis of apoptotic neutrophils both in vitro (16, 46) and in vivo (41). Previous work suggested that lipoxin-evoked increased phagocytosis was restricted to macrophages because actin cytoskeleton rearrangements occurred in THP-1 cells but not neutrophils (35), and that the phagocytosis was aimed principally at the removal of apoptotic neutrophils, since exogenous LXA4 was unable to stimulate phagocytosis of yeast particles (27). However, it has recently been shown that a stable ATL analog promotes the phagocytosis of zymosan particles and inert latex beads both in vivo and in vitro (50). Our data are in accord with the latter findings and extend the observations to the phagocytosis of bacteria via a scavenger receptor- and PI3K-dependent, opsonization-independent mechanism. Indeed, lipoxin-driven phagocytosis of apoptotic neutrophils required lectins and scavenger receptors such as CD36 (10), suggesting commonality in the mechanism of lipoxin-evoked phagocytosis of apoptotic bodies and viable bacteria by macrophages.

The finding that lipoxin-induced phagocytosis is dependent on the γ isoform of PI3K p110 is of particular interest. The phagocytosis of microbes has long been associated with PI3K activity (13), but less is known of PI3K isoform-specific effects, and even more so in the context of lipoxin signaling.
The expression of p110γ is primarily restricted to cells of hematopoietic origin (4), and its association with chemokine receptors has led to its candidacy as a potential anti-inflammatory treatment (47). However, our results suggest a role for PI3K p110 in the clearance of bacteria, and this must be taken into account before p110γ inhibition is used as a pan-anti-inflammatory strategy.

Lipoxin-induced phagocytosis of apoptotic cells by macrophages is not associated with a concomitant release of proinflammatory mediators (16). We therefore examined whether the same phenomenon could be observed when an infectious agent such as E. coli is phagocytosed. Indeed, we found that macrophages isolated from healthy donor blood produce less IL-8 and IL-1β in response to E. coli when exposed to ATL. This is not unprecedented, since lipoxin inhibition of proinflammatory cytokine release in response to a variety of stimuli has been shown. For example, LXA4 has been shown to inhibit LPS-induced TNF-α and IL-12 mRNA expression in mouse macrophages (30), IL-1β induced IL-8 production by astrocytes (8), TNF-α induced IL-8 release by alveolar and intestinal epithelial cells (5, 19), and LPS induced peroxynitrite production by human leukocytes (24). Our data differ from these earlier studies that relied on the use of specific Toll-like receptor agonists or recombinant cytokines by showing that lipoxins modulate the macrophage response to viable bacteria. This ability of macrophages to phagocytose live bacteria while simultaneously dampening their inflammatory cytokine response would allow the macrophage to play a significant role in the resolution of nonsterile inflammation, since invading microbes could be cleared from the inflamed tissue in a manner that does not lead to a significant exacerbation of the existing damage.

However, ATL was unable to inhibit E. coli-induced cytokine production when differentiated THP-1 cells were used in place of PBMC-derived macrophages. Conversely, we observed a small but significantly significant increase in IL-1β production and no change in IL-8 production. Although it is well appreciated that immortalized cell lines often display a phenotype that is not indicative of their primary cell counterparts, these results should not be discounted, particularly if the FPR2 activation elicits a proinflammatory macrophage phenotype. Phagocytosis of bacteria would result in increased exposure of proinflammatory signals to intracellular microbial sensing mechanisms that may not be inhibited by lipoxin. Mature IL-1β production is dependent on the Nalp3 inflammasome and intracellular caspases (39), whereas previous studies have indicated that the inhibition of proinflammatory cytokine release by lipoxins is dependent on inhibition of the activity of the transcription factor NF-κB (30, 57). Activation of Nalp3 by phagocytosed bacteria could therefore provide a NF-κB-independent mechanism by which inflammatory mediator production can be sustained until bacterial infections are cleared.

We also examined THP-1 macrophages for any early changes in gene expression induced by E. coli and ATL that may lead to an alteration in the antimicrobial phenotype of these cells. We assessed the change in expression following 2 h of treatment of genes involved in antimicrobial function (iNOS, MMR, CD14), antigen presentation (CD80, CD86), and chemotaxis (CXCR4, CCR7). Of these, the only gene whose expression was significantly changed by treatment with ATL and E. coli was CCR7, although it remains to be seen whether these conditions produce significant changes at the protein level. This gene is of interest because of its role in directing the migration of macrophages to secondary lymphoid tissue (17). Once again, this suggests that lipoxins play an important role in pathogen clearance that is ultimately required for the resolution of inflammation.

Given the apparent importance of macrophage-lipoxin interactions in microbe-driven inflammation, we examined whether the ability to produce or detect lipoxin is altered in CD. Initial studies found no significant differences in the expression of 5-LO, 15-LO-1 or 15-LO-2 mRNA in biopsies from patients with CD compared with controls, suggesting a normal capacity to generate LXA4. A previous study reported that patients with UC exhibit a decrease in 15-LO-2 protein expression, resulting in a decrease in colonic LXA4 production (37). This discrepancy may underscore the fact that CD and UC are distinct forms of IBD with distinguishable pathology (34), or alternatively it may be the result of our study examining mRNA expression only.

Upregulation of the lipoxin receptor FPR2 was observed in colonic biopsies from areas of active inflammation from patients with CD. Similarly, increased FPR2 has been shown in rheumatoid arthritis (20), another chronic inflammatory disease. Thus, although the biopsies revealed no evidence of increased LO gene expression, the increased receptor expression would result in enhanced lipoxin signaling. This could be due to enhanced cellular recruitment to inflamed tissue, since FPR2 is highly expressed by neutrophils, macrophages, lymphocytes, and immature dendritic cells (40). Immunofluorescence analysis of inflamed and noninflamed biopsies from patients with CD revealed that much of the immunoreactivity for FPR2 colocalized with CD68, a marker of monocytes and macrophages (22). In addition, resident cells or those recruited into an inflammatory milieu could upregulate FPR2 expression, since LPS or IFN-γ treatment of blood-derived macrophages increased FPR2 mRNA expression in vitro. These data complement those from a study of the T84 human colon epithelial cell line in which FPR2 mRNA expression was strongly upregulated by LPS, IL-1β, IL-4, IL-6, IL-13, and IFN-γ (19).

This analysis of FPR2 expression on macrophages in situ, although provocative, gives no insight into function. It is tempting to speculate that the increased FPR2 expression is a protective response in the colitic tissue aimed at disease resolution, as supported by the data showing enhanced bacterial phagocytosis, competent killing of E. coli, and a skew toward the expression of genes that would facilitate movement to secondary lymphoid tissue, and by implication impact subsequent adaptive immune responses. However, the possibility must be considered that premature termination of inflammatory reactions could allow bacteria to persist in the tissue, an event of paramount importance in the gut, which houses a vast microbiota. In fact, various pathogens are known to exploit the lipoxin pathway by producing an analog of 15-LO, leading to excess lipoxin production, which in turn promotes their escape from immunosurveillance (2, 56).

In conclusion, this study demonstrates that LXA4 potently induces macrophage phagocytosis of bacteria (and other particles) in a mannose receptor- and PI3Kγ-dependent manner. This effect would complement that of apoptotic body uptake in an inflamed tissue, allowing for tissue...
resolution after acute (or during chronic) inflammation and eradication of bacteria that have penetrated the tissue: an omnipresent concern in the gut. Support is added to this postulate with the finding of increased FPR2 expression in inflamed tissue obtained from patients with CD.

ACKNOWLEDGMENTS

The authors thank Arthur Wang and Van Phan for technical support throughout the duration of this study and Dr. P. Beck and I. Rabanni of the University of Calgary Intestinal Inflammation Tissue Bank (IITB) for assistance in obtaining cryo-tissues. We thank the Alberta IBD Consortium (funded by an Alberta Innovates-Health Solutions Team Grant) for providing infrastructure and sample acquisition by the IITB.

GRANT SUPPORT

This work was funded by a grant from the Canadian Institutes for Health Research (no. MOP-84389) to D. McKay. D. Prescott is a recipient of doctoral GRANT SUPPORT infrastructure and sample acquisition by the IITB. (funded by an Alberta Innovates-Health Solutions Team Grant) for providing distance in obtaining biopsy tissues. We thank the Alberta IBD Consortium through the duration of this study and Dr. P. Beck and I. Rabanni of the

DISCLAIMERS

The authors disclose no conflicts of interest.

REFERENCES


