Intestinal P-glycoprotein exports endocannabinoids to prevent inflammation and maintain homeostasis

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Neutrophil influx into the intestinal lumen is a critical response to infectious agents, but is also associated with severe intestinal damage observed in idiopathic inflammatory bowel disease. The chemoattractant hepoxilin A₃, an eicosanoid secreted from intestinal epithelial cells by the apically restricted efflux pump multidrug resistance protein 2 (MRP2), mediates this neutrophil influx. Information about a possible counterbalance pathway that could signal the lack of or resolution of an apical inflammatory signal, however, has yet to be described. We now report a system with such hallmarks. Specifically, we identify endocannabinoids as the first known endogenous substrates of the apically restricted multidrug resistance transporter P-glycoprotein (P-gp) and reveal a mechanism, which we believe is novel, for endocannabinoid secretion into the intestinal lumen. Knockdown or inhibition of P-gp reduced luminal secretion levels of N-acyl ethanolamine–type endocannabinoids, which correlated with increased neutrophil transmigration in vitro and in vivo. Additionally, loss of CB2, the peripheral cannabinoid receptor, led to increased pathology and neutrophil influx in models of acute intestinal inflammation. These results define a key role for epithelial cells in balancing the constitutive secretion of antiinflammatory lipids with the stimulated secretion of proinflammatory lipids via surface efflux pumps in order to control neutrophil infiltration into the intestinal lumen and maintain homeostasis in the healthy intestine.

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Intestinal P-glycoprotein exports endocannabinoids to prevent inflammation and maintain homeostasis

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Neutrophil influx into the intestinal lumen is a critical response to infectious agents, but is also associated with severe intestinal damage observed in idiopathic inflammatory bowel disease. The chemoattractant hepoxilin A3, an eicosanoid secreted from intestinal epithelial cells by the apically restricted efflux pump multidrug resistance protein 2 (MRP2), mediates this neutrophil influx. Information about a possible counterbalance pathway that could signal the lack of or resolution of an apical inflammatory signal, however, has yet to be described. We now report a system with such hallmarks. Specifically, we identify endocannabinoids as the first known endogenous substrates of the apically restricted multidrug resistance transporter P-glycoprotein (P-gp) and reveal a mechanism, which we believe is novel, for endocannabinoid secretion into the intestinal lumen. Knockdown or inhibition of P-gp reduced luminal secretion levels of N-acyl ethanolamine-type endocannabinoids, which correlated with increased neutrophil transmigration in vitro and in vivo. Additionally, loss of CB2, the peripheral cannabinoid receptor, led to increased pathology and neutrophil influx in models of acute intestinal inflammation. These results define a key role for epithelial cells in balancing the constitutive secretion of antiinflammatory lipids with the stimulated secretion of proinflammatory lipids via surface efflux pumps in order to control neutrophil infiltration into the intestinal lumen and maintain homeostasis in the healthy intestine.

Introduction

The healthy intestine is remarkable for the absence of an inflammatory response in the presence of dense microbial colonization. Maintenance of homeostasis requires a dynamic, regulated balance among the host immune response, resident microbiota, and the thin epithelial layer that separates them. While maintaining a state of noninflammation, however, the immune system must remain poised to mount an inflammatory response upon exposure to pathogens. Dysregulation of this balance can have serious consequences that may drive a variety of pathological conditions, including inflammatory bowel disease (IBD) (1). Epithelial cells serve as a physical barrier to microbial penetration and provide a sentinel system to warn immune cells of pathogen exposure by secreting neutrophil chemoattractants, placing them in a critical position to regulate the balance between pro- and antiinflammatory states. One of the first events associated with induction of a proinflammatory state is the transepithelial migration of neutrophils from the submucosal compartment to the lumen of the epithelium: a 1-way cellular-trafficking process that allows neutrophils to engage with pathogens before they can enter the host.

Neutrophil recruitment to access the epithelial lumen must occur via a carefully regulated multistep cascade of events, as the powerful effectors released by recruited neutrophils potently kill bacteria but lack specificity and thus can also cause severe collateral tissue damage. Basolateral release of IL-8 by epithelial cells stimulates recruitment of neutrophils out of the vasculature and into the lamina propria underlying the epithelial layer (1). However, IL-8 is unable to mediate the final step, in which neutrophils migrate from the basolateral (serosal) surface of the epithelium to its apical (luminal) surface. We previously identified an innate immune pathway in intestinal epithelial cells that functions to establish the chemotactic gradient required for neutrophils to move from the submucosal space to the lumen of the gut, where subsequent activation leads to inflammatory outcomes. We defined a critical role in this process for the eicosanoid hepoxilin A3 (HxA3) (2), a potent neutrophil chemoattractant that is secreted from the apical surface of the epithelium via the multi-drug resistance protein 2 (MRP2) efflux pump (3). Polarized expression of MRP2 and the activity of this MRP2/HxA3 axis are greatly increased during inflammatory states, and this pathway is nonredundant with other chemoattractants driving neutrophil recruitment, such as IL-8. Additionally, we have shown that the MRP2/HxA3 axis is a conserved mechanism at mucosal surfaces and can drive a damaging inflammatory response with severe pathological consequences (3–11). Clinical evidence further indicates that the MRP2/HxA3 pathway is acti-
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proinflammatory eicosanoid HxA3 is upregulated (13). We then demonstrated that MRP2 transports HxA3 into the intestinal lumen, establishing a chemotactic gradient biased toward the lumen that directs migration of neutrophils from the basolateral space across the epithelium, a critical step in the inflammatory process (2, 3). Having shown that this MRP2/HxA3 pathway is conserved during infection with multiple pathogens in both lung and intestinal epithelia (5, 7, 14), we sought to determine whether it also drives inflammation in the absence of infection; certain forms of IBD as well as several other clinically important human diseases (e.g., rheumatoid arthritis, psoriasis) are characterized by excessive neutrophilic inflammation with no known infectious agent.

Treatment of mice with dextran sodium sulfate (DSS) induces acute colonic inflammation characterized by epithelial damage and neutrophil influx (15). Semiquantitative liquid chromatography–dual mass spectrometry (LC-MS/MS) analysis of colonic mucosal scrapings revealed that DSS treatment strongly induced secretion of HxA3 at the epithelial surface (Figure 1A). To confirm that this increased HxA3 contributes to disease, HxA3 secretion was specifically blocked via inhibition of MRP2 using probenecid (3). We chemically conjugated probenecid to periodate-oxidized 40 kDa dextran through a reductive amidation reaction to produce a high molecular weight, hydrophilic material that would be essentially restricted to the apical surface of the intestine following its intrarectal administration in ulcerative colitis (UC) and Crohn disease (CD) patients (3), possibly due to aberrant regulation of signaling pathways. Transepithelial migration is specifically associated with diminished barrier function, and while neutrophils in the lumen may kill pathogenic microbes, their sustained activity can cause intestinal damage. In fact, disruption of the final transmigration step in mice results in the basolateral collection of neutrophils without apparent damage, emphasizing the critical nature of transepithelial migration in neutrophil-driven pathology (12). We hypothesize that the MRP2/HxA3 axis forms the proinflammatory arm of a dynamically regulated system in which inflammatory pathways that activate responses to pathogens or aberrant signals are balanced against antiinflammatory pathways that suppress neutrophil responses in the context of normal commensal colonization. We therefore investigated the mechanisms by which epithelial cells regulate transepithelial neutrophil migration during homeostasis and inflammation.

Results

We previously observed that epithelial cells dramatically increase surface expression of the membrane ABC transporter MRP2 in response to infection with a variety of pathogens including Salmonella typhimurium (3). Concurrent with this increased surface MRP2 expression, the intracellular biosynthetic pathway of the proinflammatory eicosanoid HxA3 is upregulated (13). We then demonstrated that MRP2 transports HxA3 into the intestinal lumen, establishing a chemotactic gradient biased toward the lumen that directs migration of neutrophils from the basolateral space across the epithelium, a critical step in the inflammatory process (2, 3). Having shown that this MRP2/HxA3 pathway is conserved during infection with multiple pathogens in both lung and intestinal epithelia (5, 7, 14), we sought to determine whether it also drives inflammation in the absence of infection; certain forms of IBD as well as several other clinically important human diseases (e.g., rheumatoid arthritis, psoriasis) are characterized by excessive neutrophilic inflammation with no known infectious agent.

Figure 1. HxA3 drives inflammation during DSS colitis. (A) Mucosal scrapings from C57BL/6 WT mice (n = 10) treated with 3% DSS for 7 days were enriched for lipids, and the amount of HxA3 was quantified by LC-MS/MS. *P = 0.050. (B–I) C57BL/6 mice were treated with 3% DSS for 7 days and sacrificed at day 9. Starting at day 4, daily rectal administration of PBS control (vehicle) or 1 mM probenecid conjugate was performed. **P = 0.001, Mann-Whitney 1-tailed nonparametric U test. All data are shown as mean ± SEM. n = 10 mice per group. (C and D) Paraffin-embedded sections of mid and distal colon were stained for H&E and scored by a trained investigator blinded to sample identity. *P = 0.026. Arrow highlights accumulation of neutrophils in intestinal lumen. Original magnification, ×20. (E and F) MPO activity was measured by ADHP assay over 8 minutes from (E) feces (*P = 0.044) or (F) colonic tissue and slopes calculated by linear regression (P = 0.362). For tissue, slopes were normalized to total protein content. (G–I) Total lamina propria leukocytes were isolated and stained for flow cytometry. Neutrophils were characterized as live/CD45+CD11b+Ly6G+. (G) Percentage of neutrophils (NS, P = 0.193), (H) number of neutrophils (NS, P = 0.259), and (I) representative plots of neutrophils in colon tissue.
In parallel with increased MRP2 levels, another luminal ABC transporter restricted to the luminal epithelial surface, P-glycoprotein (P-gp), is actively reduced at the epithelial surface by *Salmonella* typhimurium (16, 17), the significance of which was previously unclear. P-gp is a multidrug transporter known to efflux small neutral or positively charged hydrophobic compounds (18). Defects in P-gp expression or function are associated with IBD: decreased P-gp expression is observed in the epithelium of IBD patients, and single-nucleotide polymorphisms in the MDR1 gene encoding P-gp are associated with increased IBD risk (19–21). Further, mice lacking the *Mdr1a* gene that encodes P-gp develop spontaneous intestinal inflammation (22, 23). Evidence correlating reduced P-gp function with inflammation, combined with the hydrophobic nature of known exogenous P-gp substrates, led us to hypothesize that P-gp might secrete endogenous bioactive lipids that could serve to antagonize HxA3-mediated neutrophil transepithelial migration. We therefore analyzed the P-gp–dependent secreted lipidome of homeostatic epithelial cells to identify lipids capable of inhibiting HxA3-mediated neutrophil migration.

Supernatants collected from the apical surface of noninflamed T84 colonic epithelial cell monolayers inhibited primary human neutrophil transmigration stimulated by HxA3 in an in vitro assay; we termed the unknown compound(s) in this preparation activity modulating epithelial neutrophil discourse (AMEND). Similar spent media collected from the basal compartment of T84 cell monolayers failed to exhibit AMEND activity. Subsequent size fractionation of crude AMEND preparations using ultrafiltration demonstrated AMEND to be less than 1 kDa. An extensive series of BAKERBOND Sep-Pak extraction columns were tested to further characterize the AMEND activity. Outcomes of these matrix interaction studies suggested AMEND as having a lipophilic character (Supplemental Table 1). Notably, AMEND bound strongly to the Narc-2 matrix, used for the capture and elution of active agents in marijuana (24). The resulting optimized AMEND collection protocol involved ultrafiltration of cell supernatants to isolate compounds smaller than 1 kDa, followed by lipid enrichment using reversed-phase C<sub>18</sub> chromatography, yielding a fraction capable of inhibiting primary human neutrophil migration stimulated by HxA3 in a cell-free in vitro assay (Figure 2A). To confirm that AMEND is specifically secreted by P-gp, we created stable knockdown human T84 epithelial cell lines using shRNA to target *MDR1* and confirmed reduction of P-gp expression by Western blot (Supplemental Figure 2). Enriched supernatants from T84 epithelial monolayers were enriched for lipids and tested for the ability to inhibit HxA3-induced migration in a 56-well modified Boyden chamber assay. In order to compare across experiments with different donors, migration values within individual experiments were normalized to enriched HxA3 with vehicle treatment. For A–C, data are shown as mean ± SEM of 3 independent experiments. *P < 0.05; **P < 0.01, 1-way ANOVA. (D) Supernatants from cell lines expressing different shRNA constructs to knock down P-gp expression (B4–MDR1 and B5–MDR1). (C) Supernatants collected from control or 2 separate MDR1-knockdown cell lines (B4 and B5) were subjected to electrospray ionization MS. AEA-d8 was included in each sample to allow quantitative comparisons between samples and was used to calculate relative units of each EC. Individual ECs were normalized to the relative units in the scrambled control condition.

Figure 2. Epithelial cells secrete P-gp–dependent ECs that inhibit neutrophil migration. (A) Supernatants from T84 epithelial monolayers were enriched for lipids and tested for the ability to inhibit HxA3-induced migration in a 56-well modified Boyden chamber assay. In order to compare across experiments with different donors, migration values within individual experiments were normalized to enriched HxA3 with vehicle treatment. For A–C, data are shown as mean ± SEM of 3 independent experiments. *P < 0.05; **P < 0.01, 1-way ANOVA. (B) Superna...
receptors CB1 and CB2 as well as more recently described atypical noid receptor, suggesting that AMEND is composed of one or more observed was for agonist activity at CB2, the peripheral cannabin-

receptors (Supplemental Table 2). Importantly, the strongest signal panel, using an assay for screened for both agonist and antagonist activity against a GPCR approach to probe for receptor activation. Enriched AMEND was neutrophil transmigration, we first undertook a target-oriented enriched AMEND with FAAH completely eliminated the inhibitory activity of AMEND, whereas treatment with MAGL did not significantly affect the ability of AMEND to inhibit HxA3-induced migration (Figure 2C), suggesting that AMEND belongs to the NAE class. To confirm the specificity of enzyme treatment, we performed a similar experiment with the migration-inhibiting non-EC lipid lipoxin A4, which was not sensitive to deactivation by FAAH or MAGL (Supplemental Figure 4).

We performed MS to identify AMEND components secreted from the apical epithelial surface in a P-gp-dependent manner. First, luminal secretions were collected from polarized monolayers of T84 cells with and without verapamil treatment to block P-gp function. Unique peaks, identified by comparison of peak profiles of luminal contents of these 2 treatments separated using C18 reversed phase high-performance LC protocol, were found to have masses consistent with the H+ and Na+ adduct masses of known ECs, specifically, anandamide (AEA) (Supplemental Figure 5). We next performed a semiquantitative MS analysis, comparing supernatants obtained from control and MDRI-knockdown P-gp-deficient T84 intestinal epithe-

lial cells. Samples were spiked with deuterated AEA-d2 as an internal MS standard prior to enrichment of AMEND activity by ultrafiltration and reversed phase C8 chromatography as above prior to being subjected to high-resolution MS. H+ and Na+ adduct masses consistent with AEA, oleoyl ethanolamide (OEA), and palmitoyl ethanolamide (PEA) were reduced in the absence of P-gp expression (Figure 2D and Supplemental Table 3). These ECs all belong to the NAE class; other lipids of the MAG class of ECs, including 2-arachidonoyl glycerol (2-AG) and noladin ether (NE), were present in the basal compartment of confluent 2D human intestinal organoid cultures remained relatively constant over this same 6-hour time course, but were reduced in cultures treated with 50 μM verapamil. Relative changes in the basal compartment AEA levels were measured at 0 and 6 hours, with (white bars) and without (black bars) 50 μM verapamil addition. AEA levels are reported as normalized to time 0 values. Data are shown as mean ± SD from 3 independent experiments with 12–24 technical replicates in each experiment. *P < 0.01, unpaired t test.

Figure 3. P-gp-dependent apical secretion of ECs inhibits neutrophil migration. (A) Commer-
cially available ECs and related compounds were tested in the 96-well migration assay, with AEA, OEA, and α-LEA significantly suppressing neutrophil movement. Compounds were used at the highest concentration at which they were soluble in PBS. Data are shown as mean ± SEM of at least 3 independent experiments. *P < 0.05; **P < 0.01, 1-way ANOVA. (B) Confluent 2D human intestinal organoid cultures were used to examine apical secretion of AEA over a 6-hour time course with and without 50 μM verapamil (apical untreated, apical with verapamil), with levels being reported as average of triplicate measurements ± SD normalized to time 0 values. Increasing apical compartment levels of AEA over time were suppressed by the presence of 50 μM verapamil. Data are shown as mean ± SD from 3 independent experiments with 12–24 technical replicates in each experiment. *P < 0.01, **P < 0.001, unpaired t test. (C) The levels of AEA present in the basa compartment of confluent 2D human intestinal organoid cultures remained relatively constant over this same 6-hour time course, but were reduced in cultures treated with 50 μM verapamil. Relative changes in the basal compartment AEA levels were measured at 0 and 6 hours, with (white bars) and without (black bars) 50 μM verapamil addition. AEA levels are reported as normalized to time 0 values. Data are shown as mean ± SD from 3 independent experiments with 12–24 technical replicates in each experiment. *P < 0.01, unpaired t test.
to regulate CB2 function, highlighting the complexity of their role or roles in inhibiting HxA3-mediated neutrophil migration. Thus, further investigations are required to reveal the nature of such intricate pharmacological interactions.

Since our studies have focused on how inflammatory signals regulate the transmigration of neutrophils from the basolateral compartment to the intestinal lumen, we hypothesize that such a system requires a biased release of agents to establish a chemical gradient from the apical epithelial surface. In support of this hypothesis, we have previously shown that the MRP2 transport pump involved in regulating the efflux of HxA3 from the apical surface is restricted to this cell region to allow the establishment of such a gradient (2). Indeed, this prior work used human intestinal xenographs to show that the biology we are focusing on should be present in humans in vivo. To therefore extend the clinical significance of these findings herein, we chose to follow the pattern of AEA release, as the AMEND prototype, within cell supernatants collected from the apical or basolateral compartment of healthy human-derived organoid (colonoid) epithelial monolayers grown in a 2D format (see Methods) in the absence or presence of the P-gp inhibitor verapamil.

As shown in Figure 3B, the relative concentration of AEA increased in the apical compartment of the 2D colonoid cultures over the 6-hour study time course, but this increase was blocked by verapamil. However, AEA present in the basal compartment exhibited significant inhibitory activity, identifying these NAEs as putative AMEND components (Figure 3A). While other EC types have been reported as affecting neutrophil function in vitro (33, 34), we conclude that only NAE-type ECs are capable of specifically inhibiting HxA3-mediated neutrophil migration. Additional experiments were performed using mixtures of synthetic AEA, OEA, and α-LEA to determine their role in CB2-related effects of AMEND. Unsurprisingly, we found that these NAE-type ECs, when tested individually as well as in combinations, failed to directly act as CB2 receptor agonists; only AEA showed some activity and this was approximately 10-fold less active than 2-AG (Supplemental Table 4). Consistent with this observation, we screened for CB2 activity using NADA (a potent endogenous cannabinoid and vallinoid receptor agonist) and GP1a (a selective CB2 receptor agonist) as well as JTE 907 (a selective CB2 receptor inverse agonist); only JTE 907 showed signs of activity (Supplemental Figure 6). Taken together, these experiments reveal the possibility that AEA, OEA, and α-LEA may not function as direct CB2 receptor agonists, but rather act indirectly to regulate CB2 function, highlighting the complexity of their role or roles in inhibiting HxA3-mediated neutrophil migration. Thus, further investigations are required to reveal the nature of such intricate pharmacological interactions.

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As shown in Figure 3B, the relative concentration of AEA increased in the apical compartment of the 2D colonoid cultures over the 6-hour study time course, but this increase was blocked by verapamil. However, AEA present in the basal compartment at the...
The constant level of AEA over time in the basal supernatants suggests that the amount of AEA reaching the basal compartment is dependent on paracellular diffusion from the apical compartment and not an efflux from the basolateral surface of these 2D cultures, as we failed to observe an increase in AEA levels over this time period. Thus, there appears to be a balance between the rate of consumption and/or degradation of AEA at the basolateral surface of enterocytes and the egress of AEA from the apical compartment. Reduction in basal AEA levels in the verapamil-treated cultures likely reflects the reduced input rate to AEA via the paracellular route from the apical compartment without a coordinated change in the rate of consumption and/or degradation of AEA. Overall, these results are consistent with efflux of AEA by apically expressed P-gp, with its subsequent redistribution across the epithelium to suppress transmigration of neutrophils by direct interaction with these cells. Levels of 2-AG in the apical and basal compartment of this 2D monolayer model of healthy human intestine were below the level of detection.

A selective mechanism for EC transport out of cells had not been previously identified, although a putative membrane protein capable of bidirectional transport of AEA and 2-AG has been hypothesized (35). Having now established P-gp as an apical cell membrane efflux pump that exports NAE-type ECs in cultured epithelial cells, we sought to confirm this pathway in the intestine. Mucosal scrapings from the colons of WT and P-gp deficient (Mdr1a–/–) mice were enriched for AMEND and evaluated for their

Figure 6. CB2-deficient mice are vulnerable to severe intestinal inflammation with increased neutrophil transmigration. WT or Cnr2–/– mice were treated with 3% DSS for 7 days and sacrificed at day 9. (A) Weights are shown as percentage of day 0 (D0) weight. n = 25 WT; n = 24 Cnr2–/– mice. **P < 0.01; ***P < 0.001. (B and C) Histopathology of mid and distal colon as in Figure 1. Arrows highlight accumulation of neutrophils in intestinal lumen. *P = 0.021. Original magnification, ×20. (D) Fecal MPO activity. n = 14 WT; n = 12 Cnr2–/– mice. **P = 0.008. (E) Tissue MPO activity. n = 13 WT; n = 11 Cnr2–/– mice. NS, P = 0.471. (F) Number (NS, P = 0.482) and (G) percentage (NS, P = 0.095) of tissue neutrophils by flow cytometry analysis. (H) Representative plots of lamina propria neutrophils. (I–K) WT or Cnr2–/– mice were lethally irradiated and reconstituted with homologous or heterologous BM (donor→recipient). Following reconstitution for 7 weeks, mice were treated with DSS as above. (I) Weight curve. (J) Colon length. (K) Histopathology score. n = 7 WT→WT; n = 9 KO→WT; n = 3 WT→KO; n = 7 KO→KO mice. For all experiments, data are shown as mean ± SEM. Statistical analysis was performed with Mann-Whitney 1-tailed nonparametric U test. Histopathology scoring, MPO activity measurement, and flow cytometry analyses were performed as in Figure 1.
ability to inhibit neutrophil migration. These ex vivo scrapings from WT mice inhibited in vitro neutrophil transmigration similarly to AMEND isolated from T84 cells, whereas scrapings from Mdr1a–/– mice lacked inhibitory AMEND activity (Figure 4A). When scrapings from WT mice were pretreated with FAAH, they lost their inhibitory activity, confirming that these samples contained NAE ECs (Figure 4B). High-resolution MS revealed that similar NAE-type ECs correlated with the presence of P-gp in vitro (Figure 2D) and in vivo (Figure 4C) with the exception of PEA, which was equally abundant in samples obtained from both Mdr1a–/– and WT mice (Figure 4C and Supplemental Table 5). Although α-LEA was identified as an AMEND candidate in Figure 3B, it is not included in the LIPID MAPS database used for MS analysis, and so its potential dependence on P-gp export remains unknown.

The peripheral cannabinoid receptor CB2, which is expressed on immune cells, was identified as a potential AMEND target in our GPCR screen (Supplemental Table 2). While 2-AG is the canonical CB2 ligand, 2-AG, AEA, and other NAE-type ECs can activate CB1, CB2, and other non-CB receptors under varying conditions (36, 37). Additionally, mixed EC populations may have more complex effects, including the development of agonists or antagonists/inverse agonists when combined with other ECs (38–40). In order to determine whether CB2 signaling is required for inhibition of neutrophil migration by AMEND, we isolated neutrophils from WT and CB2-deficient (Cnr2–/–) mice. Migration of Cnr2–/– neutrophils across Salmonella-infected epithelial monolayers was significantly less impaired compared with WT neutrophil migration in the presence of enriched AMEND (Figure 5A). However, at higher concentrations, AMEND was able to partially inhibit migration of Cnr2–/– neutrophils, suggesting that signaling through receptors in addition to CB2 may contribute to AMEND activity. When experiments were performed with purified AEA, OEA, and α-LEA, CB2 expression contributed to inhibition of neutrophil transmigration by OEA and α-LEA, but not AEA (Figure 5B), although mouse neutrophils in this assay were less susceptible to AEA inhibition for reasons we cannot yet explain.

We next investigated the potential for P-gp–mediated secretion of NAE-type ECs to functionally suppress HxA3-mediated intestinal inflammation in vivo. In keeping with previous reports (23, 41), Mdr1a–/– mice, which would lack the capacity for P-gp–dependent EC secretion, developed spontaneous intestinal inflammation with age (data not shown) and were more susceptible to DSS-induced colitis.
MRP2/HxA3 axes is a mechanism by which epithelial cells them-
susceptibility of Mdr1a–/– lumen was associated with significant tissue damage that contrib-
1), neutrophil migration specifically across the epithelium into the
ing DSS-induced colitis using probenecid to inhibit MRP2 (Figure
lumen (Figure 6, C and D), while neutrophil accumulation in the
displayed dramatic increases in neutrophil influx into the intestinal
s of neutrophil function in the intestine. Unlike other antiinflam-
tion at the apical surface of intestinal epithe-

techniques, such as granulocytic myeloid–derived suppressor cells
volved in T cell tolerance, have also been shown to be involved
inflammatory events associated with IBD (51). It will be inter-
the cell, our results indicate that these efflux pumps expressed at
the apical surface of intestinal epithelial cells provide a critical link
in communication between sentinel functions of mucosal barri-
ers and the immune system. In fact, by exporting bioactive lipids
to regulate neutrophil transepithelial migration, MRP2 and P-gp
uniquely control a critical step of the inflammatory cascade (Figure
7). It should be noted that the polarized organization of the P-gp/
EC and MRP2/HxA3 axes at the apical surface of intestinal epithe-
ial cells is presumed to be critical for either suppressing or stimu-
lating neutrophil transepithelial migration. This apically restricted
organization of P-gp/EC to maintain homeostatic conditions can be rapidly reversed by apical inflammatory signal(s) through processes involving MRP2/HxA3 that are independent of neutro-
phil function in the submucosal compartment.

Discussion
Identification of a functional P-gp/EC antiinflammatory pathway that opposes the proinflammatory MRP2/HxA3 axis and controls neutrophil transmigration provides a potential explanation for sev-
eral poorly understood biological and clinical observations. P-gp
was originally identified as a xenobiotic efflux pump (45), and no endogenous substrates have heretofore been identified. Previous in vitro studies examining the impact of AEA on P-gp function were interpreted as inhibiting the function of this efflux pump (46), but these data are consistent with the possibility that AEA can function as a P-gp substrate. We now show NAE-type ECs to be endogenous substrates for P-gp. Our finding that antiinflammatory NAE secre-
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tion from epithelial cells depends on P-gp also provides a potent-

In sum, these studies have identified an antiinflammatory
P-gp/EC pathway that acts to counterbalance the proinflamma-
tory MRP2/HxA3 axis. We propose that balancing the P-gp/EC and
MRP2/HxA3 axes is a mechanism by which epithelial cells them-
selves integrate signals from the local environment to maintain
exquisite control over the initiation of neutrophil-mediated inflam-

PMR2/HxA3 and P-gp/EC signaling pathway perturbs homeostasis, leading to increased vulnerability to onset and possibly maintenance of an inflammatory state.

The development of inflammation in Mdr1a–/– mice depends
specifically on the absence of P-gp in the epithelial compartment
(22), consistent with our model in which epithelial P-gp export of
NAE-type ECs blocks inflammation. In order to confirm that the
increased susceptibility of Cnr2–/– mice to intestinal inflammation resulted from a failure of neutrophils to respond to EC inhibition, we created heterologous BM chimeras and evaluated the relative contributions of CB2 in the immune and nonimmune compart-
ments to susceptibility to DSS-induced colitis. Overall, mice trans-
planted with Cnr2–/– BM, regardless of recipient genotype, dis-
played the greatest weight loss and colon shortening, indicative of
increased inflammation (Figure 6, I and J). Transfer of WT BM into
Cnr2–/– recipients partially abrogated the inflammatory phenotype, as these heterologous mice developed less inflammation than seen in transfer of Cnr2–/– BM donors into Cnr2–/– recipient controls (Fig-
ure 6, I-K). While this experiment did not fully rule out a potential
role for CB2 in epithelial or other stromal cells, the data are con-
sistent with our hypothesis in suggesting that CB2 expression on
radiation-sensitive immune cells plays a dominant role in prevent-
ing development of colitis via inhibitory signaling by ECs.

Previous studies have demonstrated suppression of coliti-
t symptoms by CB2 agonists (42–44); demonstration of P-gp
secre tion of NAE-type ECs that can suppress neutrophil tran-
sepithelial migration, coupled with the observation that CB2-
deficient mice demonstrated an increase in this same process,
mechanistically links these findings to a specific role for NAE-
type ECs and CB receptor function during intestinal disease.
Previous studies have suggested that ECs or CB2 ligands can
reduce neutrophil migration (33, 34), but the same studies also
showed induction of neutrophil migration by AEA and 2-AG
(34), leaving the true function in vivo an open question (47). We
did not observe any chemoattractant activity of ECs alone; all of
our studies were consistent with inhibition of neutrophil tran-
sepithelial migration in the colon by NAE-type ECs that func-
tioned at least partially via CB2 agonism. The present results
may also provide a possible mechanistic explanation for anec-
dotal data from colitis patients suggesting clinical benefits from
exposure to cannabinoids (48).

The present studies have identified a new layer of regulation
of neutrophil function in the intestine. Unlike other antiinflam-
atory lipids, such as the specialized proresolving mediators that
are produced by immune cells (49), ECs appear to be secreted
locally at the apical surface by epithelial cells and can therefore
constitutively maintain an antiinflammatory environment during
intestinal homeostasis. Modulation of MRP2/HxA3 and P-gp/EC
function at the luminal surface of the intestine represents an exci-
ting new avenue for development of topical therapeutics for the
treatment of IBD. The MRP2/HxA3 pathway is conserved in infec-
tious and noninfectious lung inflammation (4, 9, 50), suggesting
that the P-gp/EC pathway may similarly regulate inflammation at
other mucosal surfaces. Additionally, other neutrophil-like cell
types, such as granulocytic myeloid–derived suppressor cells
involved in T cell tolerance, have also been shown to be involved
in inflammatory events associated with IBD (51). It will be inter-
esting to examine the potential for the P-gp/EC and MRP2/HxA3
axes described herein to regulate their actions.
Methods

Human cell lines. T84 intestinal epithelial cells at passages 50–79 (ATCC) were grown and maintained on inverted 0.33 cm² ring-supported, monolayers in 162 cm² flasks and equilibrated in HBSS +/+ at 37°C, 5% CO₂. Salmonella infection of HCT-8 epithelial cells. HCT-8 cell monolayers were grown and maintained on inverted 0.33 cm² ring-supported, collagen-coated 5 μm pore polycarbonate filters or 96-well HTS Transwell filter plates (Costar Corp.). Cells were treated apically and basolaterally with probenecid conjugate at 100 μM in HBSS with Ca²⁺ and Mg²⁺ (HBSS +/) incubated for 1 hour at 37°C. Following washing, cells were infected apically with Salmonella enterica serovar typhimurium strain SL1344 (SL) at an MOI of 375 for 1 hour. Following extensive washing, neutrophils (1 x 10⁶) were added to the basolateral surface, allowed to transmigrate across the monolayer for 2 hours, and quantified as described below.

Production of enriched HxA³. The extraction protocol and purity of HxA³ have been previously reported by the authors of this report (2, 4, 5). Briefly, to induce HxA³ efflux, Pseudomonas aeruginosa strain PA01 was grown aerobically in Luria broth (LB) broth overnight at 37°C. Cultures were washed once in HBSS +/+ and suspended at a concentration of 6 x 10⁶ bacteria/ml. H929 monolayers in 162 cm² flasks were infected for 1 hour, washed with HBSS, and then incubated in HBSS +/+ for 5 hours. Collected supernatants were captured by reverse phase chromatography on octadecylsilane (C₆) columns (Supelco; Sigma-Aldrich), washed with water, and eluted with methanol. Samples were stored at -20°C and the volume necessary for individual experiments dried down and suspended in HBSS +/+ as needed. Each new batch of enriched HxA³ was quality tested before use in experiments and was generally used after a 1:4 to 1:8 dilution.

Generation of P-gp knockdown T84 cell lines. shRNA constructs targeting the MDR1a gene were generated from a pLK0.1 plasmid background and the DNA sequences used were derived from the TRC-Hs 1.0 (human) shRNA library, which is publicly available and searchable based on the TRCN identifier at the Genetic Perturbation Platform (https://portals.broadinstitute.org/gpp/public/clone/search). Constructs are from the TRC-Hs 1.0 (human) shRNA library and are publicly available, constructs were as follows: B4 (clone TRCN00000059683), B5 (clone TRCN00000059684), B6 (clone TRCN00000059685), B7 (clone TRCN00000059686), and B8 (clone TRCN00000059687). Lentiviruses were produced by transfecting packaging cells (293T) with psPAX2, pMD.2G, and pLK0.1 plasmid constructs, using Trans-IT-LT1 lipid (Mirus Bio). After 48 hours, lentiviral supernatants were harvested, combined with 8 μg/ml polybrene (Sigma-Aldrich), and applied to T84 cells in 20% confluent monolayers. This process was repeated 24 hours later, and 48 hours following the second transfection, resistant cells were selected with 5 μM verapamil in 0.5% DMSO/HBSS +/+ in treated wells or DMSO/HBSS +/+ alone in control wells. Verapamil was added to both the apical and basal chambers, since this molecule would equilibrate natants collected over the course of 5 hours were pooled from 30 flasks, lyophilized, suspended in water, and ultrafiltered through an Amicon 1,000 Da cutoff membrane (MilliporeSigma) with N₂ positive pressure. Samples were desalted and captured on a C₁₈ Bakerbond SepPak column that was washed with water and then hexane prior to elution with methanol, dried under N₂ gas, and stored at -80°C. Before use in migration experiments, samples were suspended in HBSS +/+ as needed. Individual batches of AMEND were tested for activity in control assays before experimental use and were generally used at a 1:100 to 1:200 final dilution. For screening in the DiscoverX GPCR β-arrestin activity assay, samples were suspended in PBS to provide a x1,000 solution for screening.

Preparation of the human organoid-derived epithelial monolayers. Human intestinal epithelial cells were obtained from patients undergoing medically required surgical resections as determined by a licensed physician, as described previously (52, 53), with modifications. The present studies used cells isolated from healthy margins, as verified by a pathologist, of tissue resected from the ascending colon to obtain intestinal crypts. After washing once in ice-cold 1x PBS (Thermo Fisher Scientific), tissue strips were cut and incubated at 4°C for 30 minutes in a dissociation buffer consisting of 1x PBS, penicillin/streptomycin (Thermo Fisher Scientific), 1 mM dithiothreitol (Sigma-Aldrich), and 0.5 mM EDTA (Sigma-Aldrich). Tissue samples were then vigorously shaken to promote epithelial cell dissociation from the basement membrane, with this procedure being repeated 5 times to collect multiple fractions containing intestinal crypts that were further processed and then plated in Matrigel as previously described (53). Intestinal crypt-derived organoids were incubated in humidified 5% CO₂ at 37°C in media that consisted of a 1:1 ratio of stem cell media and I-LWRN-derived (ATCC CRL-3276) conditioned media (54). Culture medium was replenished every other day, with organoids being passaged every 7 to 9 days using standard trypsin-based techniques. Approximately 2.0 x 10⁶ cells/ml were plated in Matrigel to ensure robust organoid propagation.

Single-cell suspensions derived from organoids were plated at 1.0 x 10⁶ cells/ml on polyethylene terephthalate membrane Transwell inserts having a 0.4 μm pore size (Corning) and incubated in 1:1 stem cell/L-WRN media at 37°C with 5% CO₂ (55). Culture medium was changed every other day until confluence was reached as determined by transepithelial electrical resistance (TEER) monitoring and microscopic observation. At 48 hours prior to each experiment, the basolateral media were replenished with fresh 1:1 stem cell/L-WRN media. Medium in the apical chamber was replaced with complete DMEM plus 5 μM of the γ-secretase inhibitor IX (DAPT; Calbiochem) to promote cell differentiation (55). On the day of each experiment, monolayers were washed with 1x PBS and both the apical and basolateral media were replaced with DMEM lacking phenol red; monolayers were incubated for at least 2 hours before initiation of an experiment.

Collection of AMEND from human organoid-derived epithelial monolayers. Confluent organoid monolayers grown on polyethylene terephthalate membrane Transwell inserts in 24-well plates were washed and equilibrated in HBSS +/+ at 37°C, 5% CO₂ for 30 minutes. At the end of the equilibration step, supernatants from the apical and basolateral compartments were collected and stored at 4°C as the 0 hours samples. Liquid was replaced in both apical and basolateral compartments with 50 μM verapamil in 0.5% DMSO/HBSS +/+ in treated wells or DMSO/HBSS +/+ alone in control wells. Verapamil was added to both the apical and basal chambers, since this molecule would equilibrate.
in such a closed system. Supernatants from the apical compartment were collected and replaced with 0.5% DMSO/HBSS with or without 50 μM verapamil every 2 hours up to 6 hours to monitor the efflux of AMEND. To assess accumulation of AMEND in the basolateral chamber of this model, supernatants from the basolateral compartment were collected and replaced with 0.5% DMSO/HBSS with or without 50 μM verapamil at 0 and 6 hours. Cells were maintained at 37°C, 5% CO2, throughout the experiment. Samples were pooled from replicate wells for each compartment, apical and basolateral, and centrifuged at 1,452 g for 15 minutes at 4°C to remove cell debris. Supernatants were stored at -20°C prior to LC/MS analysis of AMEND. Data were shown as mean ± SD from 3 independent experiments with 12-24 technical replicates in each experiment. TEE values (mean ± SD) for each independent experiment are as follows: (a) 492.91 ± 88.7; (b) 441.01 ± 45.87; and (c) 447.91 ± 19.93.

96-Well neutrophil migration. Peripheral blood neutrophils from healthy human volunteers were purified from acid-citrate-dextrose anticoagulated peripheral blood by 2% gelatin sedimentation, as previously described (14). Red blood cells were removed by lysis in cold NH4Cl buffer, and neutrophils were washed with HBSS (without Ca2+ or Mg2+) and suspended to a final concentration of 5 × 107/ml. 96-Well HTS Transwell filter plates (Corning), 3 μm pore size, were coated with 0.1 mg/ml rat tail collagen and allowed to dry overnight. Enriched HxA3 (see above) was added to the lower compartment along with 0.1 mg/ml of vehicle control, enriched AMEND (see above), and neutrophil cell equivalents were calculated by comparison with sodium citrate was added to samples. MPO activity was measured, azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in 0.1 M citrate buffer (0.1 M, pH 4.2) was added, and an equal volume of 2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 0.1 M sodium citrate was added to samples. MPO activity was measured, and neutrophil cell equivalents were calculated by comparison with a standard curve, with data from individual experiments being normalized to 100% HxA3-driven migration. Data are shown as mean ± SD from 3 independent experiments with 12-24 technical replicates in each experiment. TEE values (mean ± SD) for each independent experiment are as follows: (a) 492.91 ± 88.7; (b) 441.01 ± 45.87; and (c) 447.91 ± 19.93.

Preparation of dextran conjugates. Dextran (40 kDa) was oxidized with NaIO4 in 0.1 M sodium acetate (pH 5.0). After dialysis against H2O, the oxidized dextran was reacted with excess 1,6-diaminohexane and coupled via reductive amination at pH 9.5 with the oxidation of vitamin B12, to produce a product with approximately 21 free NH2 groups per molecule of diaminohexane-modified dextran. N-(3-Dimethylamino propyl)-N′-ethylcarbodiimide (EDAC) was used to couple mannose-6-phosphate (M6P) to the oxidized dextran, with the products being dialyzed against water, lyophilized, and stored at -20°C.

Mouse experiments. C57BL/6 WT and Cnr2−/− mice were purchased from Jackson Laboratories; FVB WT and Mdr1a−/− mice were purchased from Taconic. Female mice were used at age 6 to 12 weeks, and genotypes were mixed for 2 to 4 weeks prior to experiments to equalize the genetic background.

LC/MS analysis of AMEND. MeOH-eluted preparations as above were dried under a stream of N2, suspended in MeOH/buffer, and separated by HPLC using a Vydac (Hichrom) C18 (10 um; 300 Å) semi-preparative column (10 × 250 cm). Active AMEND fractions were characterized by HPLC/MS (Genesis C18 (4 μm), 120 Å) analytical HPLC column (4.6 × 150 mm) equilibrated with 5 mM triethylamine acetic acid (pH 7.2), with the effluent analyzed by using a Finnigan LCQ Deca electrospray mass spectrometer.
microbiota. Mice were treated with 3% DSS (molecular weight 36,000–50,000; MP Biomedicals) in the drinking water for 7 days, then placed back on normal water and sacrificed at day 9, which represented peak disease state. Samples from mid and distal colon were fixed in 10% formalin, paraffin-embedded, sectioned, and stained for histopathological analysis with H&E. Each sample was graded semiquantitatively from 0 to 3 for 4 criteria: (a) degree of epithelial hyperplasia and goblet cell depletion; (b) leukocyte infiltration in the lamina propria; (c) area of tissue affected; and (d) presence of markers of severe inflammation, such as crypt abscesses, submucosal inflammation, and ulcers. Samples were scored by a trained investigator blinded to sample identity, and mid and distal colon values were averaged to give a colon histopathology score.

Isolation of neutrophils from mouse BM. Neutrophils were isolated from femurs and tibias as described in Mócsai et al. (56), with the following modifications: neutrophils were isolated in HBSS/HEPES without FCS, and red blood cell lysis was performed with ACK lysis buffer for 4.5 minutes. A total of 1 x 10^7 mouse neutrophils were added to the top well of a 96-well migration plate and allowed to migrate in the basolateral to apical direction across Salmonella-infected HCT8 epithelial cells. Neutrophil migration was quantified by ABTS assay as above.

Creation of BM chimeras. C57BL/6 WT and Cnr2–/– mice were lethally irradiated (90–100 Gy) and reconstituted with a total of 10^7 DONOR BM cells from C57BL/6 WT or Cnr2–/–. Mice were allowed to reconstitute for 7 weeks before induction of DSS colitis. Approximately 85%-90% reconstitution of donor BM was achieved in this experiment.

Isolation of lamina propria leukocytes and flow cytometry. Cell suspensions from the lamina propria were prepared as described previously (57). Intestinal tissue was cut into small pieces, treated with RPMI with 10% FBS and 5 mM EDTA to remove epithelial cells, and then incubated with 100 U/ml Collagenase Type VIII (Sigma-Aldrich) for two 1-hour periods. Cells were then applied to a discontinuous 30%/40%/75% gradient of Percoll (GE Healthcare Life Sciences) for two 1-hour periods. Cells were then treated with 100 U/ml Collagenase Type VIII (Sigma-Aldrich) for two 1-hour periods. Cells were then applied to a discontinuous 30%/40%/75% gradient of Percoll (GE Healthcare Life Sciences) and harvested from the 40%/70% interface. Cells were washed in PBS/0.1% BSA, incubated with anti-Fc receptor (αCD16/32, catalog 127609, catalog 127618), and Ly6C or Gr1 (clone RB6-8C5, catalog 101236), Ly6G (clone HK1.4, catalog 128025; and clone 30-F11, catalog 103130, 103127, 103105), CD11b (clone M1/70, catalog 104376), CD11c (clone N418, catalog 103194), and Ly6C or Gr1 (clone RB6-8C5, catalog 108407). Samples were run on a MACSquant Analyzer 10 (Miltenyi Bioscience) and analyzed using FlowJo software, version 10 (Treestar).

Analysis of MPO content in mouse samples. Samples were assayed for MPO activity as described (56). Tissue sections of colon were frozen in liquid N and stored at -80°C until use. Sections were put in hexadecyltrimethylammonium bromide (HTAB) (MilliporeSigma) buffer with lysing matrix D (MP Biomedicals) and homogenized with a Fast-Prep-24 homogenizer at level 6 for 40 seconds. Samples were combined with ADHP and fluorescence read over 8 minutes. Slopes were calculated by linear regression using Graphpad Prism and normalized to protein content for individual samples as measured by detergent-tolerant DC protein assay (Bio-Rad). For analysis of fecal samples, fecal contents were weighed and HTAB buffer added at a ratio of 10 µl/mg. Calculated slopes were used directly.

Mass spectrometric analysis of HXA3, in colonic mucosa. C57BL/6 mice were administered 5% DSS in their drinking water and sacrificed on day 7. The proximal colon from untreated or DSS-treated mice (9 mice/cohort) was harvested, and 3 intestinal segments were pooled. Mucosal scrapings were collected by scraping intestinal surfaces with a rubber policeman in PBS, and HxA content was analyzed as previously described (17).

Statistics. Data from the DSS studies (Figures 1 and 6) are reported as mean ± SEM, n = 10 mice per group, statistical significance by Mann-Whitney 1-tailed nonparametric U test. Data from the polymorphonuclear leukocyte (PMN) transmigration assays using peripheral human blood neutrophils (Figures 2 and 3A) are reported as mean ratios ± SEM of 3 independent experiments with statistical significance by 1-way ANOVA. Data from human organoid–derived epithelial monolayers (Figure 3, B and C) are reported as mean ± SD from 3 independent experiments with 12–24 technical replicates in each experiment, with statistical significance determined by unpaired (2 tailed) t test. Experiments assessing the AMEND present in mouse tissue (Figure 4) are reported as mean ± SEM from 3 independent experiments, with statistical significance determined by 1-way ANOVA. Finally, BM migration assays (Figure 5) are reported as mean ± SD from 3 independent experiments. Comparison of groups was performed by Mann-Whitney nonparametric U test. P ≤ 0.05 was considered significant.

Study approval. The care of all animals used in this study and animal experiments performed were in accordance with the University of Massachusetts Medical School IACUC (protocol 1993-12). All neutrophil-based studies were approved by the IRB of the University of Massachusetts Medical School (protocol 13006). Human organoid–derived sample collection was approved by the IRB of Massachusetts General Hospital (protocol 2015PO01908). All subjects provided informed consent prior to participation in these studies.

Author contributions. RLS, RJM, and BAM conceived and designed the study. RLS, RJM, CL, AT, ZD, AJO, BX, SR, SEF, CSF, and ALC acquired data. RLS, RJM, AL, ZD, SEF, and BAM analyzed and interpreted data. RLS, RJM, and BAM drafted the manuscript.

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