Pediatric RESEARCH

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BASIC SCIENCE ARTICLE

Indole-3-lactic acid, a metabolite of tryptophan, secreted by *Bifidobacterium longum* subspecies *infantis* is anti-inflammatory in the immature intestine

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BACKGROUND: Necrotizing enterocolitis (NEC), a necrotic inflammation of the intestine, represents a major health problem in the very premature infant. Although prevention is difficult, the combination of ingestion of maternal-expressed breastmilk in conjunction with a probiotic provides the best protection. In this study, we establish a mechanism for breastmilk/probiotic protection.

METHODS: Ultra-high-performance liquid chromatography-tandem mass spectrometry of *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) secretions was used to identify an anti-inflammatory molecule. Indole-3-lactic acid (ILA) was then tested in an established human immature small intestinal cell line, necrotizing colitis enterocytes, and other immature human enteroids for anti-inflammatory effects and to establish developmental function. ILA was also examined in immature and mature enterocytes.

RESULTS: We have identified ILA, a metabolite of breastmilk tryptophan, as the anti-inflammatory molecule. This molecule is developmentally functional in immature but not mature intestinal enterocytes; ILA reduces the interleukin-8 (IL-8) response after IL-1β stimulus. It interacts with the transcription factor aryl hydrocarbon receptor (AHR) and prevents transcription of the inflammatory cytokine IL-8.

CONCLUSIONS: This molecule produced by *B. infantis* (ATCC No. 15697) interaction with ingested breastmilk functions in a complementary manner and could become useful in the treatment of all at-risk premature infants for NEC if safety and clinical studies are performed.

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INTRODUCTION

This laboratory seeks to define the pathogenesis of necrotizing enterocolitis (NEC).[1] This condition occurs most commonly at the post-menstrual age of 28–31 weeks and results in an inflammatory necrosis of principally of the distal small intestine and the colon, leading to considerable morbidity, mortality, and medical expense.[2] We hypothesize that the condition is in part caused by an aberrant reaction of the immature intestine to colonizing bacteria.[3–5] The immature intestine is not prepared to interact with the enormous numbers of colonizing bacteria encountered after birth when the newborn enters the extrauterine environment.[6,7] We have reported that immature human intestinal cells favor inflammation over immune homeostasis with microbial–epithelial interaction.[8] Inflammation can be evoked by commensal as well as pathogenic organisms[3] in part because of a developmentally regulated innate immune response and an increased surface expression of Toll-like-4 receptors[9] as well as overexpressed signaling molecules [nuclear factor-κβ and interleukin-8 (IL-8)] and under-expressed regulatory molecules (SIGIRR, IRAK-M, and A-20).[4]

However, paradoxically it has been shown that feeding mother's expressed breastmilk or probiotics to the premature can either prevent or reduce the severity of NEC.[10,11] Although expressed breastmilk contains many passively protective factors and micro-organisms,[12] we have reported that breastmilk ingestion can stimulate the proliferation of a so-called "pioneer" bacteria over formula-fed infants that are uniquely suited for activation of protective immune responses and anti-inflammation.[13,14] We have studied the role of these "pioneer" bacteria in an ex vivo model of human premature intestine[15] and in vivo in premature mouse models.[9] A common "pioneer" bacterium associated with ingested breastmilk is *Bifidobacterium longum* subsp. *infantis* (*B. infantis*; ATCC No. 15697).[14] Feeding expressed breastmilk with probiotics is clinically the best way to prevent NEC.[16] We have shown that this bacterium and its secretions can protect against an IL-1β- (a common inflammatory cytokine in NEC) induced inflammation.[17–19] We have also shown that *B. infantis* SFs separated from the organism are anti-inflammatory and require a TLR-4 receptor to be effective.[15,18]

In this study, we extend this observation to identify the anti-inflammatory molecule in secretions as indole-3-lactic acid (ILA), a breakdown molecule of breastmilk tryptophan metabolized by *B. infantis*. In addition, this anti-inflammatory molecule is uniquely functioning in the immature intestine. We provide evidence for the isolation and identification of this anti-inflammatory molecule

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and begin to determine its anti-inflammatory mechanism in the immature human and mouse small intestine.

METHODS

Cell cultures

H4 cells, a human immature non-transformed primary small intestinal epithelial cell line characterized by our laboratory, were cultured as previously described.[20] NEC-IEC were isolated and cultured from the viable margins of resected ileal NEC tissues from a NEC neonate at 25 weeks of gestation as previously described.[9] The use of two human cell lines had the permission of Partners IRB #2011P003833 at Massachusetts General Hospital. Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as previously described.[21]

Organoid cultures

Human sample procedures were approved by institutional review board protocols IRB 1999P003833 (Brigham and Women's Hospital, Boston, MA) and IRB 2016P000949 (Massachusetts General Hospital, Boston, MA) for the derivation of immature enterospheres (FEnS). The human immature intestinal organoids were derived as described in our previous publication.[22] In this experiment the FEnS we used was from gestational ages 15 and 22 weeks therapeutically aborted fetuses as described.[22] When the culture reached confluence, it was apically treated with 5 µmol/L *N*-[(3,5-difluorophenyl) acetyl]-L-alanyl-2-phenyl] glycine-1, 1-dimethylethyl ester (DAPT) in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 for 48 h as previously described to promote cell differentiation. The monolayers were treated with 1 or 5 µM of ILA for 24 h before being stimulated with human IL-1β (1 ng/mL for 24 h) basolaterally. The supernatants from the basolateral side were collected for an IL-8 protein assay by an enzyme-linked immune sorbent assay (ELISA).

Bacterial cultures and *B. infantis* secretory fragment separation

Bifidobacterium longum subsp. *infantis* (*B. infantis*), obtained from ATCC (Manassas, VA) (ATCC No. 15697), was cultured anaerobically in a media modified from the combination of Mann–Rogosa–Sharpe (MRS) broth (Difco; BD Bioscience, Franklin Lakes, NJ) and H4 cell culture media (Supplementary Table S1).[8,18] *Bifidobacterium longum* subsp. *infantis* conditioned media at the stationary growth phase was prepared by centrifugation of probiotic cultures at 3700 r.p.m. (equal 2936 g) (Sorvall legend RT + centrifuge, ThermoFisher Scientific, MA) for 10 min at 4 °C and then by use of 0.22-µm filtration to eliminate residual bacteria. The tested filtrate was used for *B. infantis* secretory fraction (SF) separation by high-speed centrifugation (3220 × *g*, 30 min at 4 °C) (Eppendorf centrifuge 5810R, Eppendorf North America, NY) with different sizes of the Amicon Ultra centrifugal filters (MilliporeSigma, MA). The SFs (<3, 3–10, and >10 kDa) were used to test the anti-inflammatory effects in H4 cells before subjecting them to the characteristic identification.

Hydrocortisone treatment for H4 cells

H4 cells cultured in 12-well tissue culture plates to 40% confluence were treated with or without hydrocortisone (HC) (Sigma, St. Louis, MO) at 1 µM for 5 days.[23] HC, a trophic factor, has been used to convert immature cells into mature cells.[24] The cells were collected for total RNA isolation, and the aryl hydrocarbon receptor (AHR) messenger RNA (mRNA) was determined by real-time quantitative reverse transcription PCR (qRT-PCR) as described later.

Identification of anti-inflammatory effective molecules in secretory size fractions

Chemicals: Liquid chromatography–mass spectrometry- (LC–MS) grade water, acetonitrile, and acetic acid were purchased from VWR (Milan, Italy).

Instrumentation and ultra-high-performance LC–tandem mass spectrometry. Samples were filtered on a 0.45-µm nylon filter (Phenomenex®, Bologna, Italy) and directly injected into an ultra-high-performance LC–tandem mass spectrometry (UHPLC–MS/MS). Analyses were performed on a Nexera UHPLC system coupled to a hybrid ion trap-time-of-flight mass spectrometer (Shimadzu, Kyoto, Japan). For the separation of <3, 3–10, and >10 kDa *B. infantis* fractions, a Kinetex™ EVO C18 column with geometry 150 × 2.1 mm^2, 2.6 µm (Phenomenex®) was used, at a flow rate of 0.5 mL/min, with the following gradients: 0–18 min, 1–30% B, 18–19 min, 30–98% B, hold for 1.50 min, and returning to 1% B in 0.1 min. Column oven was set to 45 °C, photodiode array detection was set to 254 and 280 nm. MS detection was performed in both positive and negative electrospray ionization (ESI) mode, interface temperature was set to 250 °C, curve desolvation line: 250 °C, and nebulizer and drying gas 1.5 and 10 L/min. MS1 range 100–1500, MS/MS: data-dependent acquisition, precursors were searched in the range 100–1500 with execution trigger 10^5, collision energy 35%, and dynamic exclusion 30 s. The molecular formulas were obtained through Formula Predictor (Shimadzu). MS file was converted into mzXML format and analyzed by MZmine2. Data were deconvoluted, deisotoped, and aligned. Spectral annotation was performed against HMDB and MassBank of North America database, with max mass accuracy tolerance of 10 p.p.m. Furthermore, ultra-high-resolution MS analyses were performed on a Bruker SolariX XR FT-ICR 7 T (Bruker Daltonics, Bremen, Germany). The sample was infused by a Hamilon syringe at 2 µL/min. The instrument was tuned with a standard solution of sodium trifluoroacetate. Mass spectra were recorded in broadband mode in the range 100–1500 *m/z*, with an ion accumulation of 20 ms, with 32 scans using 4 million data points (4 M). Nebulizing (N_2) and drying gases (air) were set at 1 and 4 mL/min, respectively, with a drying temperature of 200 °C. Both positive and negative ESI were employed. Identification of compounds based on accurate MS measurements was performed by Compound Crawler version 3.0 (Bruker Daltonics). For quantitative analysis, an external calibration method was employed, a calibration curve with five levels was built, and triplicate analysis of each point was run. The UHPLC–MS/MS conditions were the same as described above. Extracted ion chromatograms of ILA MS/MS transition 204–158 were employed.

Determination of the anti-inflammatory effects of *B. infantis* SFs and ILA on H4, HC-treated H4 (H4-HC), NEC-IEC, and Caco-2 cells

H4 cells were pretreated with or without *B. infantis* SFs at the concentration of 10% or ILA (1, 5, and 20 µM) for 24 h before IL-1β stimulation (1 ng/mL) for 24 h or treated with *B. infantis* SFs or different doses of ILA alone. The IL-8 secretion into the cell culture supernatants was determined by ELISA. The effects of ILA on IL-1β-induced IL-8 secretion were also determined in H4-HC, NEC-IEC, and Caco-2 cells with the dose range (1 and 5 µM of ILA).

Determination of the anti-inflammatory effects of ILA on immature and adult mouse intestinal organ culture

C57BL/6J and TLR-4-knockout mice (Jackson Laboratory) were bred and housed in a specific pathogen-free facility. Animals were given water and standard laboratory chow ad libitum. Timed pregnant mice (embryonic day 18.5) were established as described previously.[15] Immature mice from two mouse strains and C57BL/6J (8-week old male) adult mice small intestinal tissues were collected and cut into 3-mm pieces and maintained in organ culture media as described previously.[15] After 1–2 h at 37 °C, tissues were pretreated with and without ILA (1 or 5 µM) for 24 h and then stimulated with 1 ng/mL of recombinant mouse IL-1β (R&D Systems, Minneapolis, MN) for 24 h. Supernatants were collected and stored at −20 °C for ELISA analysis. Animal procedures had been previously approved by the Massachusetts

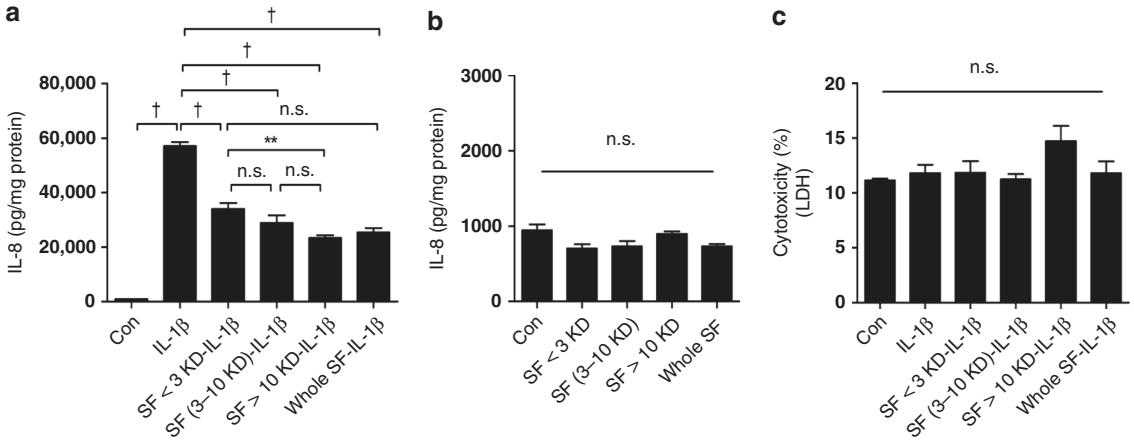

Fig. 1 The effects of different secretory size fractions of *B. infantis* on anti-inflammation and cytotoxicity in H4 cells. H4 cells were pretreated with or without *B. infantis* secretory size fractions (SFs) before IL-1β stimulation (a) or treated with SFs alone (**b**). The secretion of IL-8 (**a**, **b**) and lactate dehydrogenase (**LDH**) (**c**) into the cell culture supernatant was determined by ELISA and cytotoxicity assay—LDH assay. Graphs represent means ± SEM ($n = 3$) from three independent experiments. One-way analysis of variance (ANOVA) and Tukey's post hoc tests were used for statistical analysis (**$P < 0.01$, $^{\dagger}P < 0.001$, n.s., not significant).

General Hospital Subcommittee on Research Animal Care and Use committee (2018N000070).

Treatment with the AHR inhibitor—CH223191 in H4 cells
H4 cells were pretreated with or without various doses of AHR inhibitor—CH223191 (Sigma, St. Louis, MO) for 30 min[25] and then treated as described with ILA (1 μM) for 24 h before exposure to IL-1β (1 ng/mL) for 24 h. The inhibitor is unique for ILA and is 80% effective. IL-8 secretion into the cell culture supernatants was determined by ELISA.

Real-time quantitative RT-PCR
The total RNA of the cells was isolated by Trizol combination with Neasy RNA Isolation Kit (ThermoFish Scientific, Grand Island, NY). RNA was reverse transcribed with random hexamers using an Advantage RT-for-PCR Kit (Clontech, Mountain View, CA). The complementary DNA was amplified using iQ SYBR Green Supermix (Bio-Rad, Philadelphia, PA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were amplified in all samples to represent the housekeeper gene. The change in the normalized transcript level was expressed relative to the control sample with a change of n in CT representing a $2(^{-n})$. Primer sequences used in this study were as follows:

Human *GAPDH*: forward, 5′-ATGGGGAAGGTGAAGGTCG-3′ and reverse, 5′-GGGGTCATTGATGGCAACAATA-3′.

Human *AHR*: forward, 5′-CTTAGGCTCAGCGTCAGTTAC-3′ and reverse, 5′-CGTTTCTTTCAGTAGGGGAGGAT-3′.

LDH cytotoxicity assay
For each experiment with *B. infantis* size fragments (SFs) or ILA, lactate dehydrogenase (LDH) cytotoxic assay was performed by using a LDH Assay Kit (Roche Applied Science, Branford, CT).

IL-8 ELISA
Secreted human IL-8 and mouse IL-8 homolog protein—macrophage inflammatory protein 2 (MIP-2)—were measured by ELISA by using human IL-8 and mouse MIP-2 Detection Kits (R&D Systems, Minneapolis, MN).

Statistical analysis
All data are presented as the mean ± standard error of the mean (SEM). The unpaired Student's t test was used to compare the mean of two groups. One-way analysis of variance and Tukey's post hoc test were used to compare the mean of multiple groups. Differences of $P < 0.05$ were considered significant (*$P < 0.05$, **$P < 0.01$, $^{\dagger}P < 0.001$) (GraphPad Prism 6).

RESULTS

SFs of *B. infantis* are anti-inflammatory with H4 cells
When bacterial secretions from *B. infantis* (ATCC No. 15697) were specifically separated into different sized fractions (<3, 3–10, and >10 kDa) and exposed to H4 cells before adding the inflammatory stimulus IL-1β (Fig. 1), a highly significant reduction in IL-8 secretion was seen with all fractions (Fig. 1a). When the fractions were exposed to H4 cells in the absence of IL-1β they had no effect on inflammation (Fig. 1b), and the bacterial secretion fractions were not cytotoxic (Fig. 1c).

Identification of the *B. infantis* anti-inflammatory secretory molecule as ILA
To identify metabolites in the different molecular weight fractions of *B. infantis* secretions, we employed UHPLC coupled to high-resolution mass spectrometry in a typical untargeted workflow.[26,27] Reversed-phase (RP) chromatography ensured a good separation of both polar and nonpolar analytes. The metabolite profile of secretions revealed the presence of multiple analytes such as amino acids, sugars, dipeptides, nucleosides, glycolipids, and fatty acids. By comparison of ultraviolet–MS traces relative to the separation of cell media alone and <3, 3–10, and >10 kDa (data not shown) *B. infantis* size fractions, an intense peak possessing absorbance at 280 nm was revealed; notably, this peak was absent in the media (Fig. 2, panels a–c). The high-resolution MS spectrum showed an ion with m/z 204.0659 $[M–H]^-$ in negative ESI, with a molecular formula of $C_{11}H_{11}NO_3$. This was identified as ILA. The MS/MS fragmentation pattern and retention time (Fig. 2, panels d–h) were consistent with those reported in online spectral libraries and with its standard compound. For further confirmation, the peak was collected in a series of consecutive LC runs and analyzed by infusion through ultra-high-resolution Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), the detailed fragmentation pattern is shown in Fig. 3. The amount of ILA in the different sized fractions was comprised between 22.17 and 33.12 μg/mL. The differences in ILA content among the fractions are probably due to different mechanical properties of the molecular cutoff centrifugal filters employed for the fractionation.

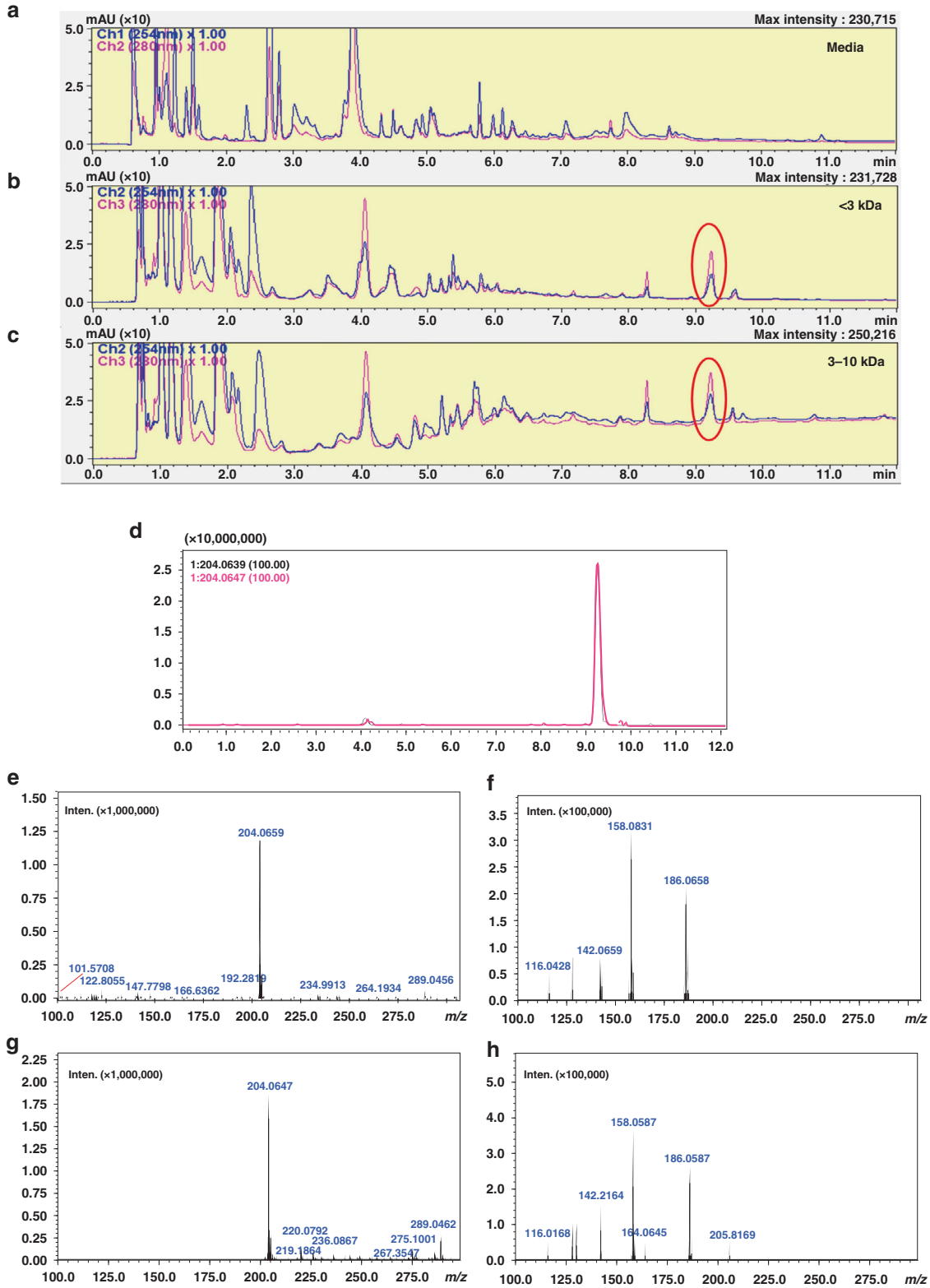

Fig. 2 Comparison of ultra-high-performance liquid chromatography ultraviolet (UHPLC-UV) profiles of the media, <3 and 3–10 kDa *B. infantis* SF, and identification of indole-3-lactic acid (ILA) as the structure of the specific peaks from *B. infantis* SF by an extracted ion chromatogram (EIC). RP-UHPLC-UV chromatograms (280 and 254 nm) showing the comparison of *B. infantis* culture media with *B. infantis* secretion size fractions 3 and 3–10 kDa. The circle shows the ILA peak, which is absent in culture media and present in both of the *B. infantis* SF (**a**–**c**). To confirm ILA, we compared the ILA standard compound retention time with the *B. infantis* 3-K fraction. **d** reports the EIC showing the ILA $[M–H]^-$ peak in *B. infantis* 3-K fractions (black line) overlapped with the corresponding standard (pink line). MS and MS/MS (**e**, **f**) spectra of ILA peak in *B. infantis* 3-K fraction compared with MS and MS/MS spectra of standard compound (**g**, **h**). The ILA identity is further confirmed by spectral matching with the online spectral libraries.

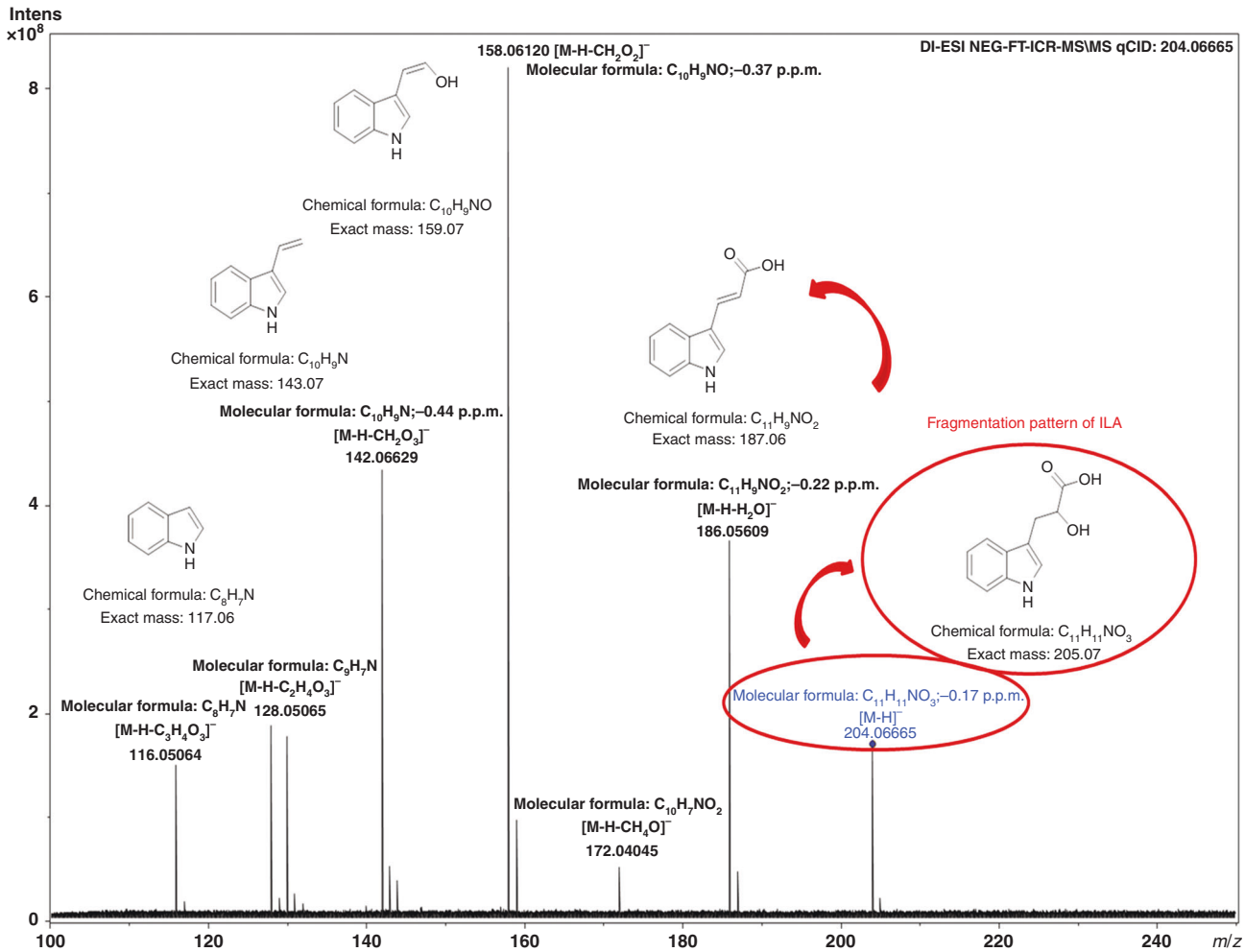

Fig. 3 ***Bifidobacterium longum* subsp. *infantis* SF structure pattern was analyzed by Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS).** Ultra-high-resolution mass spectrometry provides an accurate mass measurement and unambiguous molecular formula assignment. Thus, after isolation by RP-UHPLC of ILA peak this was infused in an FT-ICR-MS for structure elucidation. The MS/MS spectrum of isolated ILA shows its fragmentation pattern and molecular formula of each MS/MS ion with sub-p.p.m. mass accuracy.

ILA is anti-inflammatory in H4 cells

Having identified the peak in sized fractions of *B. infantis* secretions as ILA, we determined if ILA was anti-inflammatory in H4 cells. Accordingly, we incubated H4 cells with ILA before exposing them to IL-1β and then measuring IL-8 as a representative inflammatory cytokine. At levels of 1, 5, and 20 μM, ILA was significantly anti-inflammatory to the IL-1β stimulus (Fig. 4a). However, at the same doses of ILA alone, no inflammation was noted and the various doses were not cytotoxic (data not shown).

ILA is also anti-inflammatory in the small intestine from a patient with NEC and other immature enterocytes

To confirm that ILA was generally anti-inflammatory in immature intestine, we used a primary cell line isolated from resected small intestine from a NEC patient (Fig. 4b) and organoids from the therapeutically aborted human fetuses at 15 (Fig. 4c) and 22 weeks (Fig. 4d) of gestation. These enterocytes and organoids were exposed to ILA before adding IL-1β. The various concentrations of ILA resulted in a significant anti-inflammatory effect.

ILA causes anti-inflammation through an AHR and responds to TLR-4 expression

It has been reported that ILA interacts with an AHR in intestinal epithelial cells.[24,28] To determine if this receptor/transcription factor is involved in the anti-inflammatory effect of H4 cells, we determined the mRNA expression of AHR and the effect of the AHR inhibitor—CH223191 (Sigma, cat#08124, lot#0000042799) on ILA anti-inflammation after IL-1β stimulation. CH223191 is a potent and specific AHR antagonist, which inhibited AHR activator-induced AHR nuclear translocation and DNA binding with the half-maximal inhibitory concentration (IC_{50}) of 30 nM.[25] Figure 5a shows that inhibition of AHR abrogates the anti-inflammatory effects of ILA in H4 cells. We also tested ILA in TLR-4-knockout immature mice and found that anti-inflammation was lost without a TLR-4 receptor (Fig. 5b).

ILA effect is developmentally regulated

To determine if ILA was anti-inflammatory in vivo in immature versus adult intestine, we prepared C57BL/6J immature and adult mouse small intestinal organ cultures and exposed them to ILA before IL-1β stimulation. Exposure to ILA resulted in a significant reduction in IL-1β inflammation in immature mouse (Fig. 6a) small intestine, but not adult (Fig. 6b) small intestine. To determine if the effect of ILA was developmentally regulated in human intestines, we exposed ILA to H4 cells, H4 cells treated with HC (a trophic agent shown to induce maturation of immature enterocytes),[23] and to Caco-2 cells before an IL-1β stimulus (Fig. 6c–f). AHR mRNA expression was reduced in H4 cells treated with HC (Fig. 6c) and anti-inflammation of ILA was lost (Fig. 6d). AHR in Caco-2 cells was reduced (Fig. 6e) and ILA had no effect on IL-8 secretions (Fig. 6f).

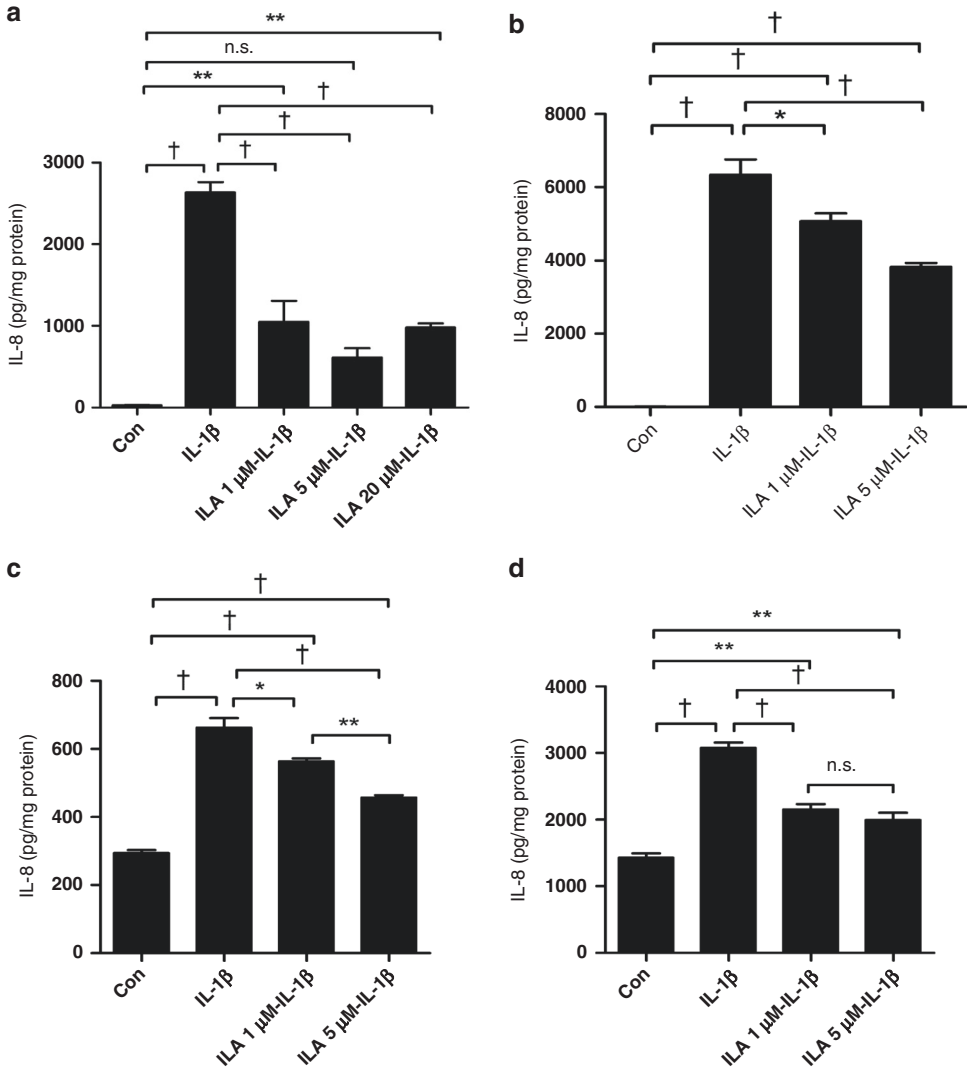

Fig. 4 The effect of indole-3-lactic acid (ILA) on anti-inflammation in H4 cells, NEC enterocytes, and immature human intestinal organoids. H4 cells (**a**), primary enterocytes isolated from resected intestine of a NEC patient (**b**), and immature human intestinal organoids isolated from therapeutically aborted fetuses at gestational ages 15 (**c**) and 22 weeks (**d**) were pretreated with or without ILA before IL-1β stimulation. The secretion of IL-8 into the cell culture supernatant was determined by ELISA ($N = 3$). Graphs represent the mean ± SEM from three independent experiments. One-way analysis of variance (ANOVA) and Tukey's post hoc tests were used for statistical analysis (*$P < 0.05$, **$P < 0.01$, $^{\dagger}P < 0.001$, n.s., not significant).

Proposed mechanism for ILA effect on immature enterocytes

Taken together, we propose that ILA, a breakdown product of the amino acid tryptophan, present in large amounts in breastmilk, is produced by *B. infantis* metabolism and mediates the mechanism of anti-inflammation in immature human enterocytes (H4, NEC primary cell lines, and immature enteroids). ILA requires an interaction with TLR-4 and with the AHR receptor to interfere with its transcription of the inflammatory cytokine IL-8 that causes excessive inflammation in the premature intestine resulting in NEC (Supplementary Fig. S1).

DISCUSSION

We have previously reported that *B. infantis* (ATCC No. 15697) and its secretions were anti-inflammatory in ex vivo experiments using a human immature primary small intestinal cell line (H4 cells) and in vivo in immature mouse small intestine,[15,18,19] suggesting that this "pioneer" bacterium known to exist as part of the initial colonizing microbiota of breastfed newborns and breastmilk itself potentially functions in concert to impart protection against the severe intestinal inflammatory necrosis leading to NEC.[29,30] We have reported that size fractions of *B. infantis* secretions (5–10 kDa) when exposed to immature enterocytes were protected against a lipopolysaccharide-stimulated inflammatory response in a primary human small intestinal cell line.[18] We know from clinical studies that increased levels of IL-1β are found in the intestinal secretions of premature infants developing NEC.[31] We have also reported that anti-inflammatory secretions from *B. infantis* require an increased enterocyte surface-level expression of TLR-4 receptors[9,32] and affect the developmentally expressed transcription factor, AP-1, to interfere with IL-8-stimulated inflammation.[17] More recently, by using transcription profiles of RNA isolated from H4 cells exposed to *B. infantis* secretions followed by an IL-1β inflammatory stimulus, we have determined that the transcription of tight junction genes (occludin and claudin) is increased and intercellular transport is reduced.[21]

In this study, we extend these observations to determine the structure of the molecules in secretions, which mediates

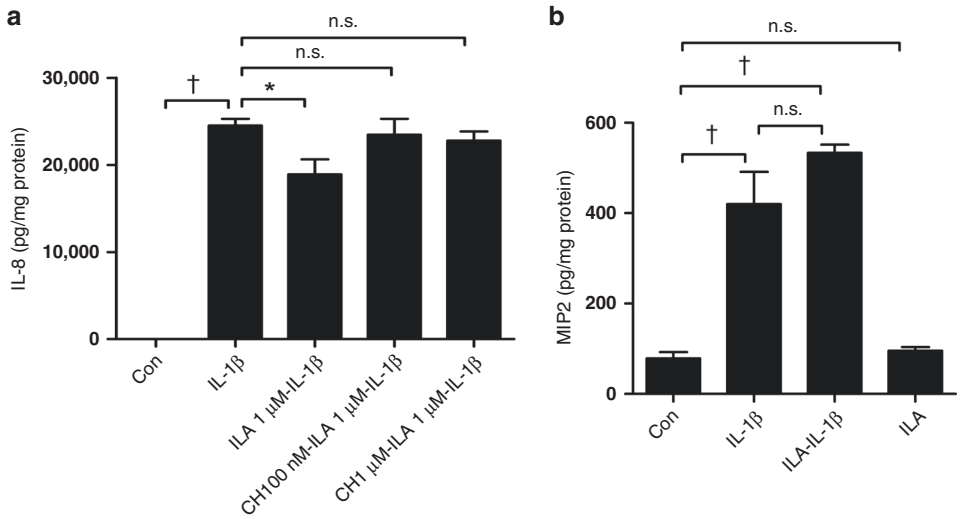

Fig. 5 ILA anti-IL-1β-induced IL-8 induction is mediated by aryl hydrocarbon receptor (AHR) in H4 cells and TLR-4 is required for ILA anti-inflammation in immature intestine. a H4 cells were pretreated with or without different doses of AHR inhibitor—CH223191 and then treated with ILA before exposure to IL-1β. The secretion of IL-8 into the supernatant was determined by ELISA**. b** TLR-4-knockout immature mice (embryonic day 18.5) small intestinal organ cultures were pretreated with or without 5 μM of ILA before IL-1β stimulation. The secretion of microphage inflammatory protein 2 (MP2) into the supernatant was determined by ELISA. Data are represented as the mean ± SEM, $n = 3$ from three independent experiments for **a** and $n = 6$, from the repeated experiments for **b**. One-way analysis of variance (ANOVA) and Tukey's post hoc tests were used for statistical analysis (*$P < 0.05$ and $^{\dagger}P < 0.001$, n.s., not significant).

enterocyte anti-inflammation. We compared the metabolite profiles of cell media with the 3, 3–10, and >10 K fractions after UHPLC–MS/MS analysis (Fig. 2).

The accurate mass spectrometry measurement provided the molecular formula: $C_{11}H_{11}NO_3$ and by tandem MS fragmentation, assisted by spectral library matching, it was finally identified as ILA (Fig. 3).

It is important to recognize that all strains of bifidobacteria studied are capable of producing ILA from tryptophan,[33,34] but *B. infantis* represents a unique bacterium present in breastfed newborns. ILA is only anti-inflammatory in immature intestine and breastmilk contains tryptophan capable of producing ILA.[35] This suggests that *B. infantis*, a known probiotic in infants, in combination with expressed breastmilk may be an important way to prevent NEC.

When ILA was incubated with H4 cells at the concentration found in secretions and at higher concentrations before a stimulus with IL-1β, the molecule was anti-inflammatory ex vivo in H4 cells (Fig. 4a), in a primary cell line from resected small intestine in a patient with NEC (Fig. 4b), and additionally in immature intestinal organoids (Fig. 4c, d), suggesting that this was a generalized effect in immature enterocytes.

Since ILA is a potential ligand for the AHR in enterocytes, we determined the effect of ILA in immature enterocytes in which AHR was inhibited. We found that the inhibitor of AHR caused the anti-inflammatory effect to be lost, suggesting a pathway for anti-inflammation in immature enterocytes (Fig. 5a). When we used ILA and IL-1β in immature mice with the TLR-4 receptor knocked out, ILA also lost the anti-inflammatory effect, suggesting that TLR-4 present in abundance on the surface of immature enterocytes was involved in the anti-inflammatory process (Fig. 5b).

The evidence that ILA was only effective as an anti-inflammatory molecule in immature intestine existed in both mouse models and human enterocytes. We exposed wild-type immature and adult organ cultures of mouse intestine to ILA before an inflammatory stimulus with IL-1β (Fig. 6a, b). The anti-inflammatory effect was only present in the immature mouse. In addition, we measured the AHR gene in immature enterocytes treated with HC to cause intestinal maturation and Caco-2 cells. The receptor genetic level was high in immature enterocytes and lower in cortisone-treated immature enterocytes and Caco-2 cells. In like manner, when ILA and IL-1β were used in the cortisone-treated and Caco-2 cells, they lacked an anti-inflammatory response strongly suggesting that ILA is only anti-inflammatory in immature but not adult intestine.

Tryptophan is a substrate for intestinal bacteria leading to important metabolites. The health of the patient has become very important as investigators have addressed microbial–epithelial crosstalk.[36] Of particular importance is the indole metabolites that use as ligands AHR as a translational receptor in enterocytes and immunocytes.[37] This is particularly true in the early life when bifidobacteria are abundant in breastfed babies and in experimental animals have been shown to influence development of immunologic defense.[38] It has also been associated with inflammatory bowel disease. Low tryptophan levels and a decrease in AHR in active patients have been reported,[39] and indole metabolites of tryptophan have been used in animal models to treat colitis.[35] In like manner, the role of tryptophan metabolite deficiency has been associated with irritable bowel syndrome and metabolic syndrome. Furthermore, decreased levels of AHR receptors have been found in patients with multiple sclerosis.[40]

It has previously been reported that a number of colonizing intestinal bacteria, particularly Gram-negative organisms, can also metabolize the amino acid tryptophan to produce metabolic pathways whose production improves health and provides immunologic protection.[33,34] One such pathway utilizing indole metabolites (ILA, indole-3-pyruvic acid (IPA), etc.)[33,34] uses the AHR to activate the immune system and other intestinal functions for purposes of homeostasis. For example, IPA, an AHR agonist, can be used to inhibit inflammation associated with animal models of colitis.[35] AHR is a ligand-activated transcription factor stimulated by dietary and microbial metabolites that plays an important role in maintaining homeostasis by intestinal mucosa. ILA is a metabolite of tryptophan.[33]

Lactic acid bacteria are capable of converting amino acids into secondary metabolites. This process starts with a deamination step, which in the case of aromatic amino acids is operated by the enzyme aromatic aminotransferase that converts amino acids into

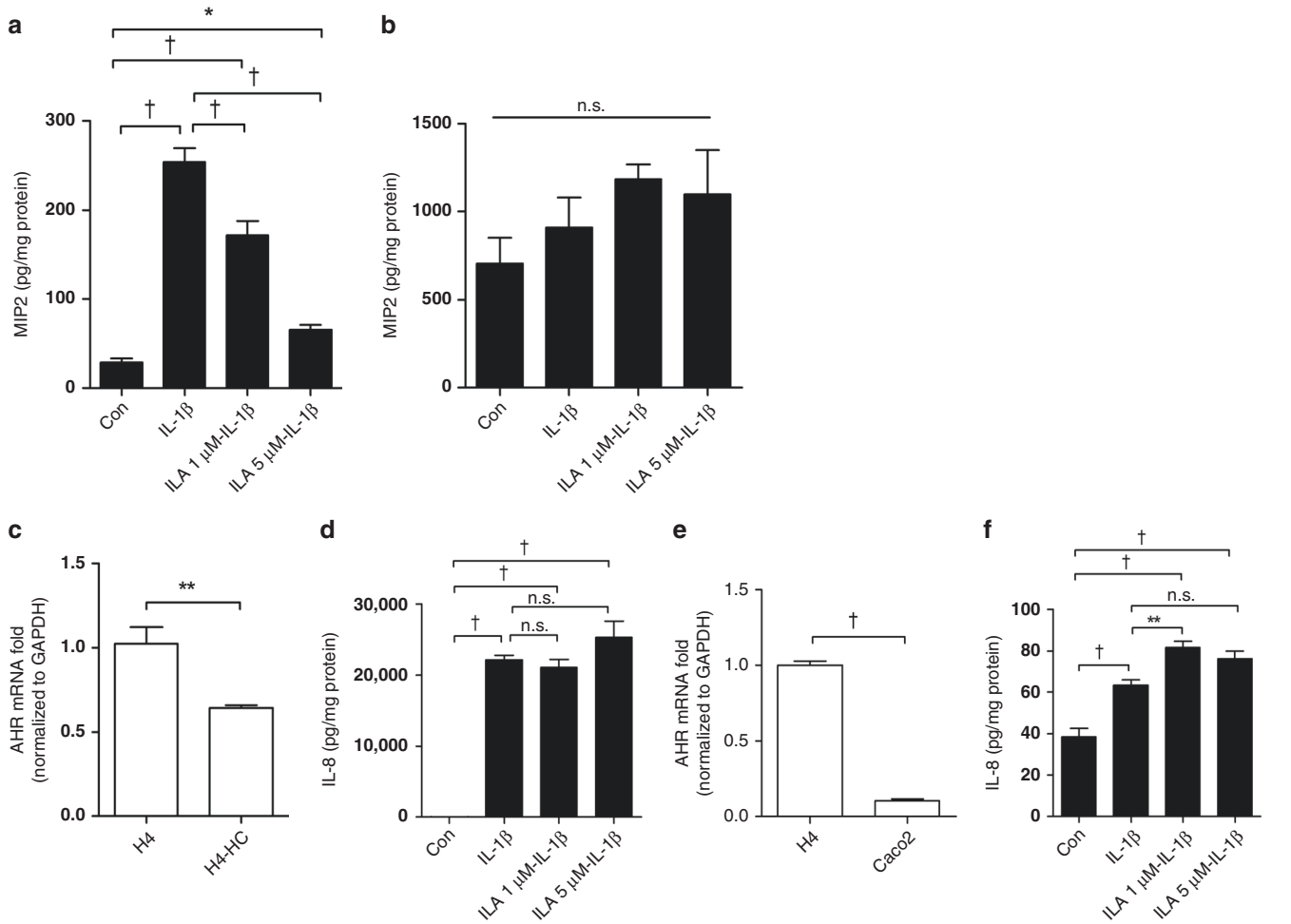

Fig. 6 AHR and ILA anti-inflammatory responses to IL-1β are developmentally regulated. C57BL6 (**a**) immature (embryonic day 18.5) and (**b**) adult (8-week-old male) mice small intestinal organ cultures were pretreated with or without ILA before IL-1β stimulation. The secretion of MP2 into the supernatant was determined by ELISA. **c** Expression of AHR mRNA in H4 cells and hydrocortisone-treated H4 (HC-H4) cells**. d** IL-8 reaction in HC-H4 cells' exposure to ILA before an IL-1β stimulus. **e** AHR mRNA in H4 and Caco-2 cells. **f** IL-8 secretions in Caco-2 cells pretreated with or without ILA before IL-1β stimulus. Data are represented as the mean ± SEM, $n = 6$ for mice and $n = 3$ for cells (from three independent experiments). *T* test was used for two-group and one-way analysis of variance (ANOVA), and Tukey's post hoc tests were used for multiple-group statistical analysis (*$P < 0.05$, **$P < 0.01$, $^{\dagger}P < 0.001$, n.s., not significant).

α-ketoacids. These compounds are further converted by dehydrogenases into hydroxy acids. The presence of ILA has also been reported in *Lactobacillus casei* and *L. helveticus*,[33,36] and this molecule has shown anti-inflammatory activity in keratinocytes against UV-B-induced damages.[33] Increased amounts of these tryptophan metabolites were found in other bacterial species such as *Megamonas hypermegale*, *Roseburia intestinalis*, *Ruminococcus obeum*, *E. rectale*, and so on.[36]

We know that breastmilk, particularly colostrum, has high levels of the essential amino acid tryptophan.[11,12] We also know that ingestion of maternal-expressed breastmilk in premature infants is protective against NEC.[11,12,14] It has also been reported that breastmilk-induced bacteria (*B. infantis*, *Lactobacillus acidophilus*, and *Bacteroides fragilis*) known as "pioneer" bacteria[14] are specifically stimulated with initial colonization with ingested breastmilk in premature infants compared with probiotics given to formula-fed infants.[13] They can function together with probiotics to protect against the expression of NEC in premature infants.[13,16]

Based on the results presented here, we hypothesize that *B. infantis*, a known "pioneer" bacterium,[13,14] present in the intestine of premature infants fed mother's expressed breastmilk, metabolizes breastmilk tryptophan to produce ILA, which acts to inhibit the transcription factor's (AHR) stimulation of IL-8 only in the immature intestine that expresses TLR-4 on its surface and thus protects against the excessive intestinal inflammation seen in the premature infant and thought to be a contributing factor in NEC. Supplementary Figure S1 is a cartoon that depicts this proposed mechanism of anti-inflammation in immature enterocytes. A previous clinical study has shown that "pioneer" probiotics are most effective in preventing NEC when given with expressed breastmilk.[16] Thus, this observation provides a mechanistic explanation to support the additive effect of breastmilk and probiotics in the prevention of NEC. Additional in vivo studies in animals are required, and safety studies in humans before a large-scale, single-protocol clinical trial with ILA can be performed to determine efficacy in immature intestine.

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AUTHOR CONTRIBUTIONS

D.M. oversaw all experiments; E.S., P.C. and E.S. analyzed the *B. infantis* fractions and identified ILA; K.G., K.D. and W.Z. contributed to ELISA and LDH assay analysis, cell line maintenance, and mouse experiments; W.A.W. conceived of the research plan and analyzed experimental data.

ADDITIONAL INFORMATION

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