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Local Generation of Kynurenines Mediates Inhibition of Neutrophil Chemotaxis by Uropathogenic Escherichia coli

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During epithelial infections, pathogenic bacteria employ an array of strategies to attenuate and evade host immune responses, including the influx of polymorphonuclear leukocytes (PMN; neutrophils). Among the most common bacterial infections in humans are those of the urinary tract, caused chiefly by uropathogenic Escherichia coli (UPEC). During the establishment of bacterial cystitis, UPEC suppresses innate responses via multiple independent strategies. We recently described UPEC attenuation of PMN trafficking to the urinary bladder through pathogen-specific local induction of indoleamine 2,3-dioxygenase (IDO), a tryptophan catabolic enzyme previously shown to have regulatory activity only in adaptive immunity. Here, we investigated the mechanism by which IDO induction attenuates PMN migration. Local tryptophan limitation, by which IDO is known to influence T cell longevity and proliferation, was not involved in its effect on PMN trafficking. Instead, metabolites in the IDO pathway, particularly L-kynurenine, directly suppressed PMN transepithelial migration and induced an attached, spread morphology typical of PMN both at rest and in the presence of chemotactic stimuli. Finally, kynurenines represent known ligands of the mammalian aryl hydrocarbon receptor (AHR), and UPEC infection of Ahr−/− mice recapitulated the derepressed PMN recruitment observed previously in Ido1−/− mice. UPEC therefore suppresses neutrophil migration early in bacterial cystitis by eliciting an IDO-mediated increase in local production of kynurenines, which act through the AHR to impair neutrophil chemotaxis.
cretion of toxins and other exoproteins, bacterial pathogens may activate host anti-inflammatory pathways in order to survive and persist. We recently demonstrated that uropathogenic Escherichia coli (UPEC; the chief cause of community onset and health care-associated UTIs), in contrast to nonpathogenic E. coli, induced local expression of the mammalian IDO1 gene (encoding indoleamine 2,3-dioxygenase), both in vitro and in a murine model of bacterial cystitis. In turn, local activation of IDO was found to inhibit PMN trafficking into infected bladder tissue (23). PMN therefore represent the first identified innate cellular components regulated by IDO activity. In addition, these results illustrate how early induction of IDO by UPEC arriving in the bladder leads to local immune suppression, providing a window of opportunity for UPEC to establish a protected niche by invading superficial epithelial cells of the bladder (24, 25). This niche then provides a haven for rapid bacterial replication while protecting the burgeoning bacterial colony from neutrophils that are subsequently recruited to the bladder in response to multiple chemoattractants.

In order to further define the mechanism by which induction of IDO suppresses PMN recruitment and thereby enhances UPEC survival early in infection, we utilized several models of host-pathogen interaction. We show that, rather than tryptophan limitation, it is local production of kynurenine metabolites that attenuates PMN migration across the infected uroepithelium. These kynurenines exhibited a direct effect on PMN motility, as demonstrated by morphological analysis of adherent neutrophils in vitro. Furthermore, the derepressed PMN influx into the infected bladder that we observed in Ido1−/− mice (23) was here recapitulated in mice lacking expression of Ahr, implicating this transcriptional regulator in control of PMN chemotaxis. In total, our data further elucidate an emerging role for IDO in innate cellular defense and microbial pathogenesis.

MATERIALS AND METHODS

Reagents. Fibrinogen (Fbg; catalog no. F4883), l-tryptophan (TRP; catalog no. T8491), l-kyurenine (KYN; catalog no. K8625), 3-hydroxy-kynurenine (HK; catalog no. H1771), 3-hydroxyanthranilic acid (HAA; catalog no. 148776), CH-223191 (catalog no. C8124), F-Met-Leu-Phe (FFLP; catalog no. F3506), 1-methyl-α,α-tryptophan (1-MT; catalog no. 860646), dimethyl sulfoxide (DMSO; catalog no. D5879), and fetal bovine serum (FBS; catalog no. F2442) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human tumor necrosis factor alpha (TNF-α) was purchased from R&D Systems (Minneapolis, MN).

Bacterial strains and culture. Uropathogenic Escherichia coli (UPEC) isolate UTI89 was isolated from a patient with cystitis (26), while MG1655 is a type 1 piliated K-12 nonpathogenic strain of E. coli (27). For infection of mammalian cells in vitro, bacteria were grown overnight in standing Luria-Bertani (LB) broth at 37°C. Cells were pelleted by centrifugation and resuspended in sterile phosphate-buffered saline (PBS) to an optical density at 600 nm (OD₆₀₀) of approximately 1.0 and further diluted in RPMI 1640 to achieve a multiplicity of infection (MOI) of approximately 40:1 bladder cell or 10:1 PMN.

Murine cystitis. C57BL/6, Tnf−/− (The Jackson Laboratory), or Ahr−/− (kind gift of K. Moley) female mice, 8 to 10 weeks of age, were transurethrally inoculated with ~2 × 10⁷ CFU of UPEC strain UTI89, and bladders were harvested and homogenized at the indicated time points (detailed protocols are published in references 25 and 28). All animal experiments received prior review and approval of the Animal Studies Committee at Washington University School of Medicine. To quantify tissue myeloperoxidase (MPO) activity, a measure of neutrophils present in bladder tissue (29), bladder homogenates stored at −80°C were thawed and cleared by centrifugation, and the lysates were analyzed using a Fluoro MPO kit (Cell Technology, Mountain View, CA) per the manufacturer’s instructions.

Cell culture and isolation. A human bladder epithelial cell line (5637, HTB-9; American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS. Peripheral blood neutrophils were purified from the venous blood of healthy volunteers by dextran sedimentation and density gradient centrifugation as described previously (29), with informed consent and in accordance with a protocol approved by the Washington University Human Research Protection Office. Independent experiments utilized PMN from different adult donors.

Transwell chemotaxis assay. A model of PMN transepithelial migration has been described previously (29–31). Briefly, inverted uropathelial cell layers were grown to confluence on Transwell permeable supports (Costar catalog no. 3472; Corning, New York, NY) (pore size, 3 μm). The cells were washed with RPMI 1640 and mock infected or infected for 1 h with 60 μl of bacterial suspension (E. coli UTI89 strain MG1655 at an MOI of approximately 40:1) in RPMI medium with TRP (200 μg/ml) or an appropriate DMSO vehicle control as indicated in Results and in the figure legends. Following infection, the inserts were transferred to a 24-well plate in which the lower reservoir contained 0.6 ml of RPMI 1640, with TRP (200 μg/ml), fMLF (100 nM), KYN (0.125 to 12.5 μM), fMLF (100 nM) plus KYN (0.125 to 12.5 μM), or an appropriate vehicle control as noted. Approximately 10⁶ neutrophils were then added to the upper reservoir in 100 μl RPMI 1640, with TRP (200 μg/ml) or an appropriate DMSO vehicle control as indicated. Samples were incubated at 37°C in 5% CO₂ for 1 h to allow transepithelial migration. The top chambers were removed and rinsed, and neutrophils in the lower reservoir were enumerated using a hemacytometer. Neutrophil transepithelial migration is expressed as a percentage of input cells (counted from control wells bearing no Transwell insert). Statistically significant differences were assessed using an unpaired Student t test.

Cytotoxicity assays. The effect of kynurenine treatment on euakaryotic cell integrity was assessed by the use of a fluorescent assay for lactate dehydrogenase (LDH) release (CytoTox-ONE; Promega, Madison, WI) according to the instructions of the manufacturer. Approximately 10⁶ neutrophils were incubated in a 96-well plate for 1 h at 37°C in 5% CO₂ with RPMI 1640 alone or RPMI 1640 containing the indicated concentrations of KYN or vehicle control. Relative fluorescence was measured in a microplate reader (Synergy 2; BioTek, Winooski, VT), and percent cytotoxicity was calculated in comparison to the maximum fluorescence measured with detergent-lysed cells.

Immunofluorescent staining and morphology analysis. Approximately 1.5 × 10⁷ neutrophils were plated on Fbg-coated glass coverslips (10 μg/ml) and incubated in the presence of fMLF (100 nM), KYN (12.5 μM), fMLF plus KYN, TNF-α (25 ng/ml), TNF-α plus 1-MT (10 μM), or a medium-only control as indicated for 30 min at 37°C in 5% CO₂. Cells were fixed, permeabilized, stained for actin with rhodamine phalloidin (1:1000), and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1:50,000) as described previously (32). The coverslips were sealed with an anti-fade reagent (ProLong Gold; Life Technologies) and imaged using a 63× oil-immersion lens on a Zeiss Axioskop 2 MOT Plus fluorescence microscope. Idential exposure times were used for each sample within an individual experiment, and images were acquired with an Axioamc MRM Monochrome camera using Axiosvision software. At least 10 random fields of view were imaged after selection with the DAPI channel only, and the morphology of all cells in each image for each condition was assessed (50 to 150 total cells per condition in each of 3 to 4 independent experiments). Images were numbered to blind the analyst to the treatment group. Small, symmetrical cells with no protrusions were categorized as “round.” Elongated cells with asymmetric actin staining and protrusions in a single direction were categorized as “polarized.” Cells that assumed a larger diameter on the cell circumference, and exhibited symmetric actin staining were catego-
cDNA, and relative levels of target expression were calculated by the gene was normalized to that seen with the endogenous GAPDH con- baseline corrections. The transcript abundance of the first-strand cDNA was synthesized using random primer hexamers (RNeasy minikit; Qiagen) according to the instructions of the kit manufacturer. RNA integrity was assessed by spectrophotometry, and isolation sufficient RNA for gene expression analysis. Approximate total RNA was isolated by silica membrane bind-

Gene expression analysis. The abundance of CYP1A1 transcript has been used as a measure of aryl hydrocarbon receptor activation under various conditions (32). Cultured bladder epithelial cells or human PMN were treated in 24-well plates, and the contents of 4 wells were pooled to isolate sufficient RNA for gene expression analysis. Approximate total numbers of cells per condition were 106 for bladder epithelial cells and 107 for PMN. Conditions included KYN (12.5 μM), KYN (12.5 μM) plus CH-223191 (10 μM), or vehicle-only (DMSO) treatment for 2 h at 37°C in 5% CO2. Cell lysates were prepared and homogenized (Qiagreader; Qiagen, Valencia, CA). Total RNA was isolated by silica membrane binding (RNEasy minikit; Qiagen) according to the instructions of the kit manufacturer. RNA integrity was assessed by spectrophotometry, and first-strand cDNA was synthesized using random primer hexamers (Superscript II; Life Technologies). Real-time PCR was performed in a 10-μl reaction mixture containing approximately 0.1 μg template cDNA, 1× TaqMan assay mix (CYP1A1 assay identification [ID] no. Hs00153120_m1, GAPDH [glyceraldehyde-3-phosphate dehydroge-nase] gene assay ID no. Hs99999905_m1; Life Technologies), and TaqMan Fast PCR master mix (Life Technologies) on an Applied Biosys-
tems 7500 Fast instrument (standard cycling conditions: 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C) using SDS v1.4 software with automatic threshold and baseline corrections. The transcript abundance of the CYP1A1 target gene was normalized to that seen with the endogenous GAPDH control gene, and relative levels of target expression were calculated by the threshold cycle (ΔCt) method (33), where fold change in expression is equal to 2^ΔΔCt. Data are presented as fold change in transcript abundance under the experimental condition relative to the mock-treated cal-

RESULTS
UPEC suppression of neutrophil migration is not mediated by tryptophan limitation. We reported previously that epithelial infection with UPEC (but not with nonpathogenic E. coli) induces local expression of the tryptophan catabolic enzyme IDO1, resulting in suppression of PMN migration in both in vitro and in vivo models of the early inflammatory response (23). To determine if this immunomodulatory mechanism was dependent on local alteration in tryptophan availability (as reported for T lymphocytes [7]), we measured the effect of tryptophan supplementation on PMN transepithelial migration in vitro. We observed that excess tryptophan did not restore PMN transepithelial migration across UPEC-infected epithelial layers (and similarly had no effect on migration in response to nonpathogenic E. coli; Fig. 1).

Kynurenine metabolites inhibit transepithelial neutrophil migration. While suppression of neutrophil migration was not due to tryptophan limitation, the possibility remained that an increased local concentration of the IDO1 reaction product (namely, L-kynurenine) or of one or more of its downstream metabolites was important for this effect. To test this hypothesis, we measured neutrophil transepithelial migration upon the addition of L-kynurenine or one of several metabolites downstream in the kynurenine pathway of tryptophan degradation (Fig. 2). Starting concentrations of kynurenine metabolites were based on supernatant concentrations after UPEC infection of cultured bladder epithelial cells (10 to 15 μM; data not shown) and on observations with multiple other cell types (see, e.g., references 34, 35, and 36). The addition of L-kynurenine (KYN) or 3-hydroxykynurenine...
(HK) resulted in a significant decrease in PMN transepithelial migration toward the chemoattractant fMLF compared to the vehicle control results (Fig. 3A). Neutrophil migration upon addition of an equivalent concentration of 3-hydroxyanthranilic acid (HAA) trended similarly but did not reach statistical significance (Fig. 3A). In addition, the suppressive effect of L-kynurenine was dose dependent (Fig. 3B). Of note, the measured effect of L-kynurenine on neutrophil migration in this in vitro model was not due to neutrophil cytotoxicity, as determined by a standard assay for membrane integrity (Fig. 3C).

Kynurenines Impair PMN Chemotaxis. Because neutrophil polarization and rearrangement of the actin cytoskeleton are critical to the process of neutrophil chemotaxis, we characterized neutrophil morphology on a fibrinogen-coated surface in response to L-kynurenine in the presence or absence of the chemoattractant fMLF. The majority of unstimulated PMN were small and round in appearance, with minimal, evenly distributed f-actin staining (Fig. 4; Untreated). Upon exposure to fMLF, neutrophils have been shown to exhibit a polarized morphology featuring localization of f-actin filaments to the leading edge or pseudopod, correlating with chemotactic activity (31, 37). Our fMLF treatment also yielded this expected cellular morphology (Fig. 4; fMLF). In contrast to fMLF, treatment with L-kynurenine alone resulted in a significant percentage of cells adopting an attached, spread morphology (Fig. 4; KYN). Furthermore, L-kynurenine treatment in the presence of fMLF resulted in a significant decrease in the percentage of polarized cells and a significant increase in the percentage of spread cells compared to fMLF treatment alone (Fig. 4; fMLF+KYN). These data suggest that kynurenine directly alters neutrophil morphology and impairs chemotaxis.

In addition, the spread morphology of kynurenine-treated
PMN was reminiscent of that observed upon neutrophil treatment with TNF-α, previously proposed to provide a “stop signal” that counters PMN chemotaxis (38). As TNF-α has been demonstrated to induce IDO activity in various mammalian cell types (reviewed recently in reference 39), we wondered whether TNF-α-stimulated human PMN were allowed to adhere on fibrinogen-coated slides in unmodified RPMI 1640 (Untreated) or in RPMI 1640 with TNF-α (25 ng/ml) or TNF-α plus 1-MT (10 μM), with processing and morphological classification as round, spread, or polarized as described for Fig. 4. Data represent the means and SEM of the results of 3 experiments performed with different donors. TNF-α treatment induced a predominantly spread morphology (**, P < 0.01) (versus Untreated), which was unaffected by 1-MT treatment (ns, not significant).

The kynurenine-responsive aryl hydrocarbon receptor participates in IDO modulation of neutrophil function. It has been previously shown in other cell types (e.g., T cells and brain tumor cells) that L-kynurenine is an endogenous ligand for the aryl hydrocarbon receptor (AHR) (13, 14). AHR-ligand interaction is complicated, in that ligands can behave as agonists, antagonists, or both depending on context (40); furthermore, receptor stimulation and downstream effects are dependent on cell type and participation of other components in a multimeric transcription factor complex (18). We sought first to demonstrate a relationship between L-kynurenine treatment and cellular expression of the canonical AHR-responsive CYP1A1 gene (32), which encodes a P450-associated hydroxylase acting on aryl hydrocarbons. In both human bladder epithelial cells and primary neutrophils, the normalized abundance of CYP1A1 transcript was upregulated upon L-kynurenine treatment; this response was prevented by the addition of a specific inhibitor of AHR (CH-223191; Fig. 6A). In order to demonstrate a role for AHR in modulation of neutrophil function, we introduced UPEC into the bladders of AHR-deficient mice and measured bladder tissue MPO levels, a surrogate for neutrophil influx (23, 29), at 6 hpi. As we observed previously in Ido1−/− mice (23), PMN influx (as reflected by tissue MPO content) was significantly higher in
AHR-dependent induction of local IDO1 activity (23). In the present study, we demonstrate to increase neutrophil recruitment to the lungs of influenza virus-previously identified PMN migration suppressor YbcL (51, 52) does not rely on chemotaxis. Finally, our data do not discount the possibility that suppression of PMN migration by kynurenines is also mediated in parallel via mechanisms distinct from AHR.

The observed alteration in PMN morphology upon kynurenine treatment recapitulates a PMN phenotype reported previously with TNF-α treatment. Specifically, TNF-α was shown to initiate a p38 mitogen-activated protein kinase (MAPK)-dependent "stop signal" that induces neutrophil adhesion and not polarization, resulting in diminished PMN motility toward chemotactants (38). Existing data indicate that UPEC induces local TNF-α secretion during cystitis (21, 50), and we show that in the bladder as a whole, TNF-α influences early IDO induction in response to UPEC (Fig. 5A). However, our present data (Fig. 5B) indicate that TNF-α effects on neutrophil chemotaxis are not attributable to IDO induction, as chemical inhibition of IDO did not abrogate the TNF-α "stop signal" (Fig. 7). Another piece of this model that remains to be specified is the UPEC-specific effector or attribute that promotes IDO activation; of note, the recently identified PMN migration suppressor YbcL (51, 52) does not rely on IDO for its action (unpublished data).

In summary, our findings support the idea of an expanded role for IDO in innate cellular responses through the AHR-mediated effects of kynurenine metabolites on neutrophil function, in addition to the previously identified roles in adaptive immune regulation. Beyond this, our data illuminate a mechanism by which a prototypic bacterial pathogen leverages these innate host pathways to facilitate the establishment of epithelial infection.
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