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TITLE: Mechanisms and Therapeutic Implications of the Pregnane X Receptor Targeting Indole Bacterial Metabolites in Inflammatory Bowel Disease

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14. ABSTRACT

This proposal addresses a significant medical problem, namely, infection triggered inflammation in the intestines (technically called post-infectious inflammatory bowel disease) in military personnel. Compromised gut barrier integrity is an important risk factor that contributes to the onset of IBD, especially post-infection. The environmental cues and its molecular controls regulating intestinal barrier function are poorly understood in homeostatic and pathophysiologic states like infection-induced IBD. Our studies show a novel direct link between intestinal microbial metabolism (i.e. specific microbial metabolites) and regulation of intestinal permeability via a pathway regulated by an orphan nuclear receptor, PXR, and TLR4. We demonstrate that in the small intestines (which mirrors what happens in large intestines), where PXR is expressed in intestinal epithelial cells in a crypt-villus gradient, in homeostasis, dietary tryptophan-derived bacterial metabolites (i.e. indoles and indole metabolites in particular indole 3 propionic acid or IPA) tonically activate PXR and induce a down-regulation of the Toll-like Receptors, in particular TLR4, and its downstream signaling pathway. This results in modulating the abundance of TNF-α, which in turn modulates intestinal barrier function (i.e. permeability). In the context of an inappropriate increase in inflammatory signals (e.g., infection), suppression of PXR, and/or excess loss of dietary modulators (e.g., tryptophan), and/or specific indole metabolizing bacteria (e.g., antibiotics) results in increased permeability, thus exacerbating underlying disease predisposition and pathology. In this model, restitution of signaling homeostasis, either by reconstituting intestinal loss of indole-metabolite producing bacteria and/or PXR activating bacterial metabolites (i.e. IPA), could result in abrogating pro-inflammatory signals and loss of barrier permeability in the context of intestinal inflammation. Our proposal will address the role of these metabolites and of PXR in maintaining barrier function in infection induced colitis in mice.

15. SUBJECT TERMS

Inflammatory Bowl Disease, PXR, Macrophages

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INTRODUCTION:

This proposal addresses a significant medical problem, namely, infection triggered inflammation in the intestines (technically called post-infectious inflammatory bowel disease) in military personnel. Compromised gut barrier integrity is an important risk factor that contributes to the onset of IBD, especially post-infection. The environmental cues and its molecular controls regulating intestinal barrier function are poorly understood in homeostatic and pathophysiologic states like infection-induced IBD. Our studies show a novel direct link between intestinal microbial metabolism (i.e. specific microbial metabolites) and regulation of intestinal permeability via a pathway regulated by an orphan nuclear receptor, PXR, and TLR4.

We demonstrate that in the intestines where PXR is expressed in intestinal epithelial cells in a crypt-villus gradient, in homeostasis, dietary tryptophan-derived bacterial metabolites (i.e. indoles and indole metabolites in particular indole 3 propionic acid or IPA) tonically activate PXR and induce a down-regulation of the Toll-like Receptors, in particular TLR4, and its downstream signaling pathway. This results in modulating the abundance of TNF-α, which in turn modulates intestinal barrier function (i.e. permeability). In the context of an inappropriate increase in inflammatory signals (e.g., infection), suppression of PXR, and/or excess loss of dietary modulators (e.g., tryptophan), and/or specific indole metabolizing bacteria (e.g., antibiotics) results in increased permeability, thus exacerbating underlying disease predisposition and pathology. In this model, restitution of signaling homeostasis, either by reconstituting intestinal loss of indole-metabolite producing bacteria and/or PXR activating bacterial metabolites (i.e. IPA), could result in abrogating pro-inflammatory signals and loss of barrier permeability in the context of intestinal inflammation. Our proposal will address the role of these metabolites and of PXR in maintaining barrier function in infection induced colitis in mice. The immunologic implications of PXR in the pathogenesis of intestinal injury during infection, is unknown. Furthermore, the role of PXR in the pathogenesis of infection-induced colitis is unknown. Furthermore, the therapeutic mining for metabolite mimics is unexplored. Thus, we hypothesize that PXR is critical for regulating (abrogating) the inflammatory response in both epithelial and innate immune cells such as intestinal macrophages, and thus important for limiting pathology following enteric infection. We further hypothesize that the combinatorial binding of indole(s) to the human PXR LBD (ligand binding domain) can be chemically mimicked (bacterial metabolite mimicry) towards discovery of more potent new chemical entities and drugs that activate PXR and repress inflammation. The main goal of our lab (Khanna Lab) is to investigate the role of PXR, in the maintenance of intestinal immunological homeostasis in vivo under steady state conditions or after an enteric infection or other inflammatory cues.

For our studies we will use several different mouse models (including the PXR^{flx} –Villin-cre, LysM-cre, CD169-cre mice and wild-type littermates) to analyze differences under steady state and following infection with intestinal pathogens such as *C. Rodentium-stx and salmonella* bacterial load, spread, translocation and mucosal immune responses before and after infection. These studies will uncover whether IEC intrinsic PXR regulates inflammation and through which key cytokines/inflammogens. The ultimate goal of understanding fundamental biology is to develop novel and unconventional approaches to curing disease (e.g., metabolite mimics for IBD therapy). The short-term goal is to further the knowledge of a novel cellular pathway dictated by PXR in post-infectious IBD.

KEYWORDS: *Intestinal epithelial cells, Inflammatory bowel disease, macrophages, innate immune cells, Pregnane X receptor, colitis*

ACCOMPLISHMENTS:

The accomplishments are outlined below and the data are included in this section.

Our major goal of the project:

Aim 1. To investigate the role of PXR, in the maintenance of intestinal immunological homeostasis in vivo under steady state conditions or after an enteric infection:

What was accomplished under these goals?

To this end, we first began by determining the status of the macrophage subsets and other myeloid cells in the intestines of mice that were PXR deficient or normal. Since, the PXR-floxed mice are still being crossed to villin-cre, CD169-cre or LysM-Cre, we began our studies using global PXR deficient mice.

To determine the function of PXR in regulating enteric infection, we began our studies with infecting PXR WT and PXR deficient mice with wildtype *Citrobacter rodentium* orally. *Citrobacter rodentium* shares approximately 67% gene homology with *Escherichia coli* (E.coli). Given that mice are resistant to E.coli infection, *Citrobacter rodentium* is a valid model to study enteric E.coli infections. Both PXR WT and PXR deficient mice were monitored for weight loss as well as fecal shedding of bacterial. To this end, we sterilely collected fecal samples from each mice every 2 days post infection. The fecal sample was weighed and resuspended in sterile 1xPBS and homogenized. Fecal slurry was then serially diluted and plated on MacConkey Agar plates to allow for selection of gram-negative and enteric bacilli bacterium.

15 days post infection, both groups were euthanized and the colon and caecum was harvested for gross anatomy analysis for colonic hyperplasia. Additionally, the distal colon was fixed with PLP and froze in OCT for histopathological and confocal microscopy analysis. 20um thick sections were cut and imaged using antibodies against CD169, CD11c, O152, and EpCAM. A Zeiss 880 confocal microscope was used to acquire the images. Imaris software was used to analyze and quantify imaging data.

As shown in Fig.1, PXR deficient mice exhibit weight reduction following *Citrobacter rodentium* infection compared to infected PXR WT control mice. Additionally, as shown in Fig.2, PXR deficient mice has a higher propensity to be colonized by and clearance of the bacteria compared to PXR WT mice. These data suggest that PXR has a functional role in regulating the clearance of enteric *Citrobacter rodentium* infection and the subsequent weight regulation. Interestingly, we did not observe any gross colonic hyperplasia difference between PXR WT and PXR deficient mice (Fig.3), which is typically indictive of an IBD phenotype. However, the lack of differences in gross colonic hyperplasia between the groups could be a result of both groups exhibiting equal levels of colonic hyperplasia. Therefore, to better determine the overall level of disease pathogenesis, we conducted confocal microscope of 20 um thick sections to determine crypt hyperplasia, which is another measure of IBD phenotype characterized by over active intestinal stem cell proliferation. In contrast to our gross anatomy observation, we found that PXR deficient mice have increase crypt length compared to PXR WT mice as shown in Fig.4.

To further confirm colonization of both PXR WT and PXR deficient mice, we conducted immunofluorescent staining of colon cross-sections against O152 antigen, which is present on E.coli and *Citrobacter rodentium*. As shown in Fig.5, both PXR WT and PXR deficient mice were both colonized by *Citrobacter rodentium* and consistent with our fecal shedding and crypt hyperplasia data, PXR deficient mice exhibit greater immune-reactive staining for O152 antigen compared to PXR WT. Additionally, it is observed that in the PXR KO mice, *Citrobacter rodentium* is able to translocate further down the crypt and lamina propria compared to PXR WT, where majority of the bacteria resides in the luminal space.

Next we observed the in situ phenotype of myeloid cells (dendritic cells and CD169+ macrophages) between PXR KO and PXR WT following infection. We found that PXR KO mice exhibit cluster of CD11c+ dendritic cells closer to the luminal space (Fig.6) compared to PXR WT where the CD11c+ dendritic cells appear to be equally distributed along the laminal propria. Additionally, with respect to CD169+ colonic macrophages, we found that greater clustering around the basement membrane closer to the muscularis (Fig.7). These data suggest a differential role of CD11c+ dendritic cells and CD169+ colonic macrophages following enteric infection in the absence of PXR.

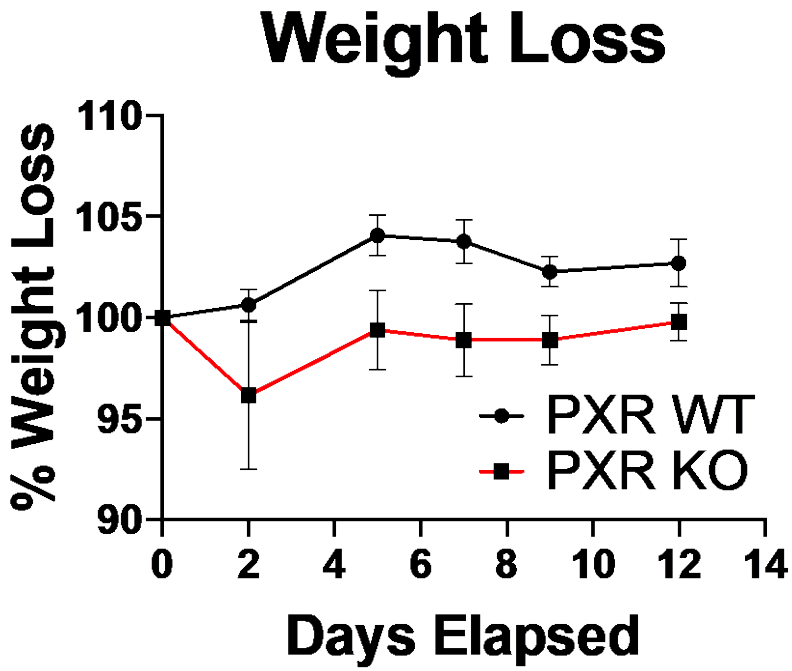

Fig.1. Weight loss between PXR WT and PXR KO mice following 2 x 10^9 CFU *Citrobacter rodentium DBS100* oral infection. N = 5 mice/group

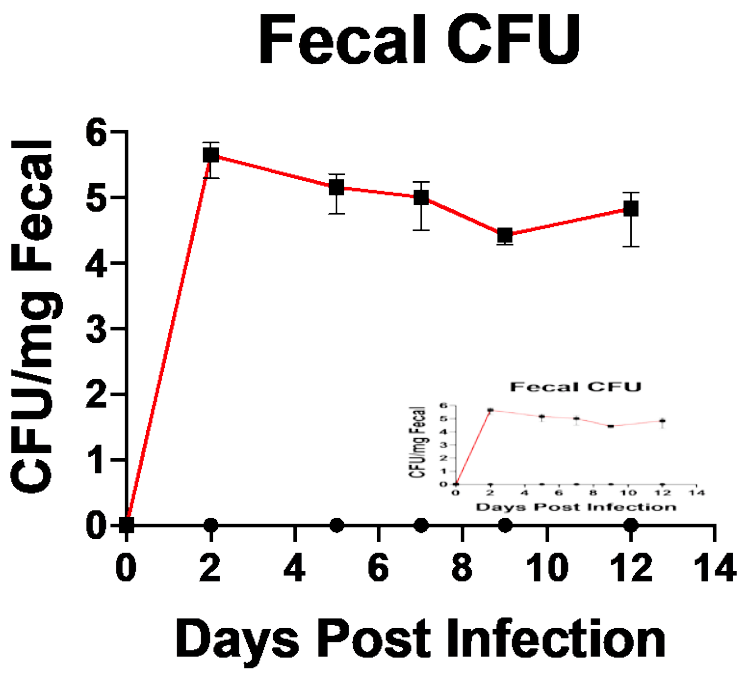

Fig.2. Fecal shedding of *Citrobacter rodentium DBS100* between PXR WT and PXR KO mice. N = 5 mice/group

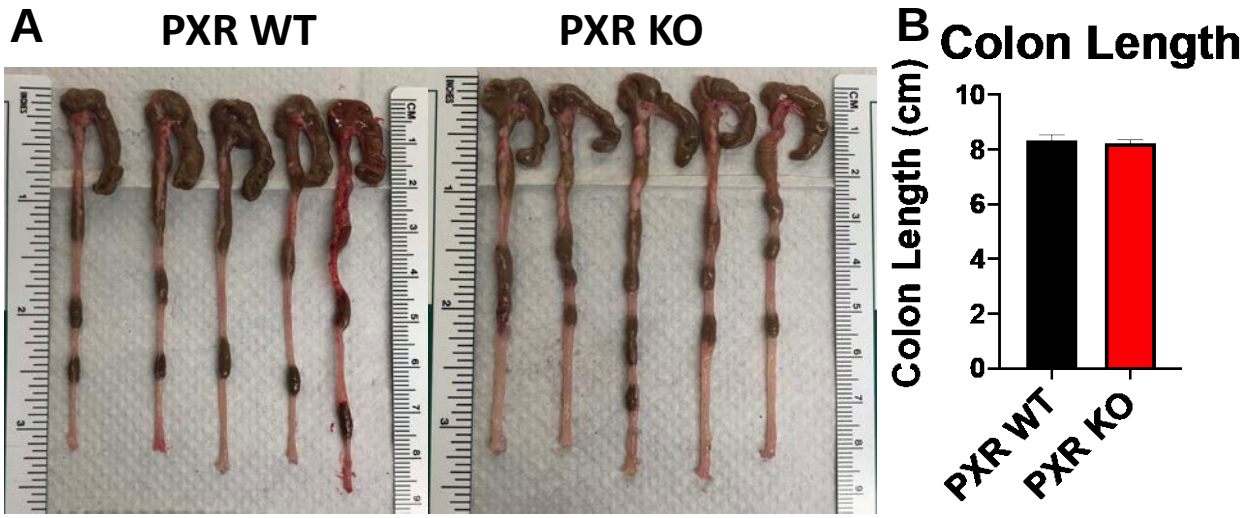

Fig.3. A) Gross anatomy of PXR WT (left) and PXR KO (right) colon 15 days post infection with *Citrobacter rodentium DBS100* infection. B) analysis of total colon length. N = 5 mice/group

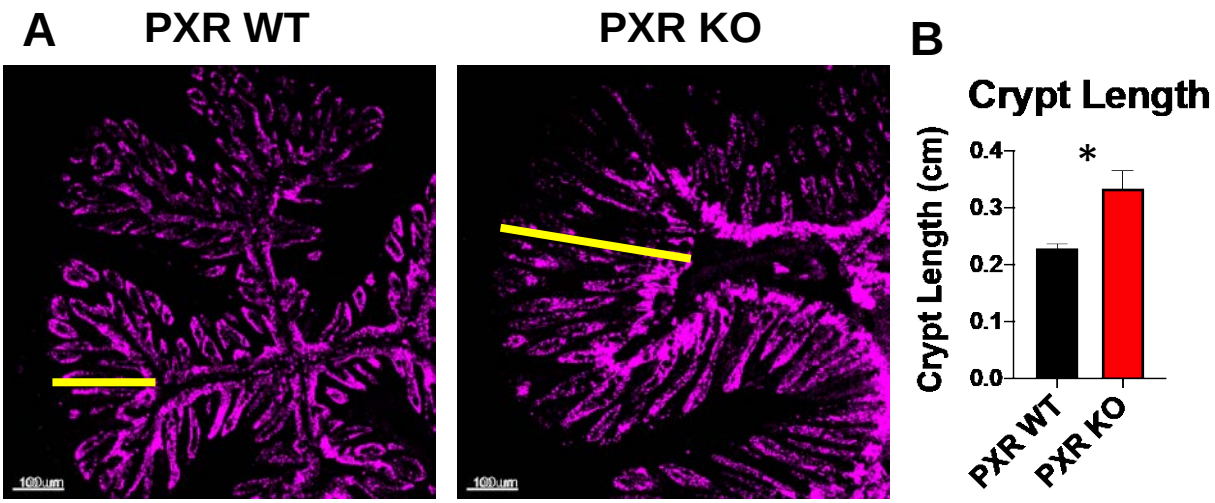

Fig.4. A) Confocal images of PXR WT (Left) and PXR KO (Right) colon cross-sections immunostained with anti-Epcam (purple) for epithelial cells 15 days post infection with *Citrobacter rodentium DBS100* infection. B) Analysis of crypt hyperplasia. Student T-test, * p<0.05. N = 5 mice/group

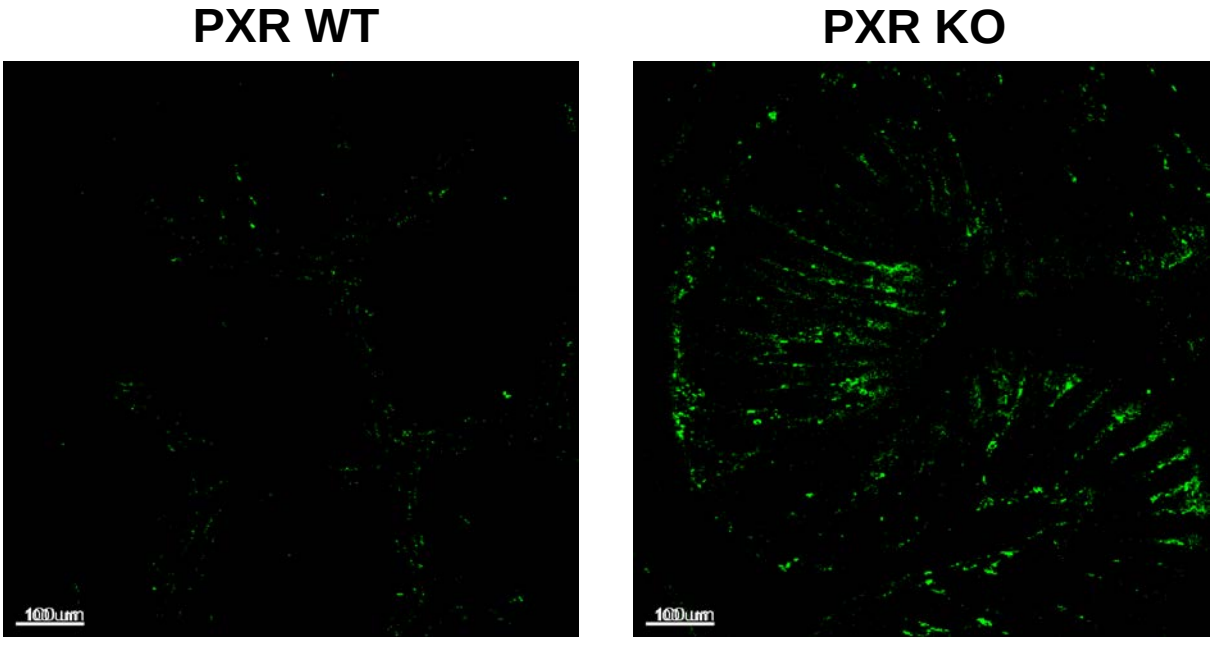

Fig.5. Confocal images of PXR WT (Left) and PXR KO (Right) colon cross-sections immunostained with anti-O152 antigen (green) for *citrobacter rodentium* 15 days post infection with *Citrobacter rodentium DBS100* infection. N = 5 mice/group

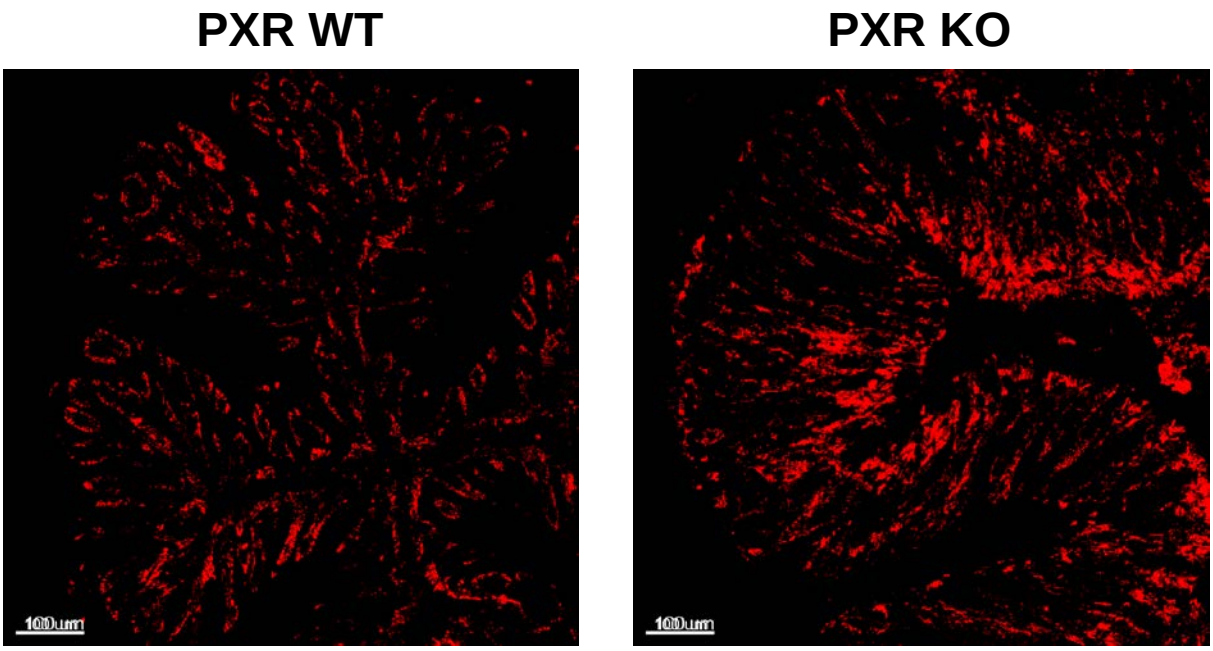

Fig.6. Confocal images of PXR WT (Left) and PXR KO (Right) colon cross-sections immunostained with anti-CD11c (red) for colonic dendritic cells 15 days post infection with *Citrobacter rodentium DBS100* infection. N = 5 mice/group

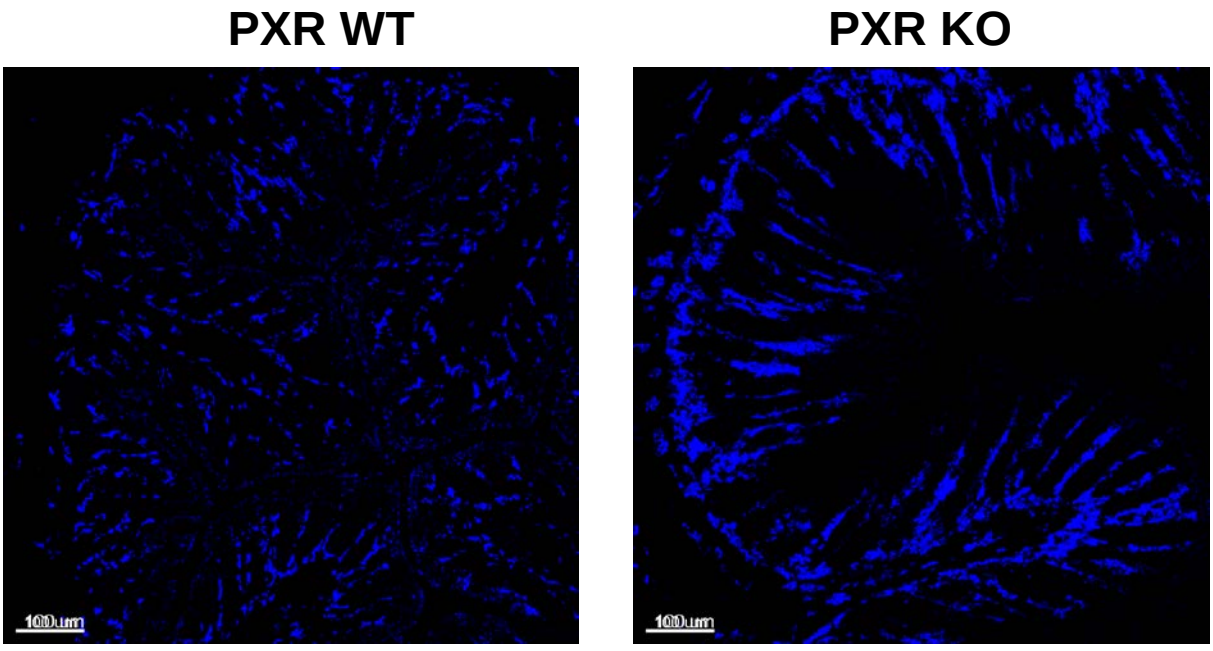

Fig.7. Confocal images of PXR WT (Left) and PXR KO (Right) colon cross-sections immunostained with anti-CD169 (blue) for colonic CD169+ macrophages 15 days post infection with *Citrobacter rodentium DBS100* infection. N = 5 mice/group

What opportunities for training and professional development has the project provided?

- Jennifer Rutowski received further training in confocal imaging and multicolor flow cytometric analysis. She continues to attended several training sessions for lab and animal handling safety. She has also been trained on novel multicolor imaging platform called CODEX that uses the newly acquired Keyence microscope.
- Graduate Student Stephen Yeung was partially funded by this grant before he received a fellowship and was put on a T32 training grant. However, he has continued to work on this project.

How were the results disseminated to communities of interest?

The studies are ongoing and it is too early for publications and thus, nothing to report yet. I have presented part of this work in local and national seminars.

What do you plan to do during the next reporting period to accomplish the goals?

Although my move from University of Connecticut to NYU delayed the acquisition of some of our PXR mutant mice due to the requirement of rederivation of the PXR KO mice and the PXR floxed mice. We now have the PXR floxed mice that have been crossed to CD169-Cre and Villin-Cre. The crossing took additional time to obtain PXR-floxed-homozygous/Cre-hets. We have now begun and will continue to characterize the epithelial and macrophage intrinsic mechanisms by which PXR regulates homeostasis in the gut mucosa after enteric infection. We have begun testing the double and triple transgenic mice for their efficacy and ensuring that we do not have any leaky transgene.

Our infection studies demonstrate a novel mechanism by which PXR may wield its immune-modulatory effects in the intestines. We will now test the PXR dependent mechanism by which these macrophages or other immune or stromal cells in the gut help clear intestinal pathogen and regulate inflammation.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Until now the data generated above confirms our hypothesis that PXR has an important role in regulating inflammation in the gut and this regulation likely involves innate immune cells. The current model that we can propose is that CD169+ macrophages regulate the recruitment of neutrophils and monocytes that cause immunopathology in the gut. We found this to be true even in steady state conditions. Inflammatory cues such as intestinal infection will only exasperate this effect. Thus, targeting this macrophage subset may be a novel therapeutic strategy against IBD. In addition, our latest findings show that infection induced inflammation, as well as the localization and clearance of intestinal pathogens may regulate the outcome of disease (IBD) onset and chronicity of inflammation. PXR in macrophages (and perhaps intestinal

epithelial cells) appears to regulated this process, which is a novel finding.

What was the impact on other disciplines?

- Nothing to Report

What was the impact on technology transfer?

- Nothing to Report

What was the impact on society beyond science and technology?

- Nothing to Report

CHANGES/PROBLEMS:

Changes in approach and reasons for change

- Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

- Nothing to Report

Changes that had a significant impact on expenditures

- No, we do have slightly more funds available at the end of the year that will be carried over and used right away in the next year.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

- Nothing to Report

Significant changes in use or care of human subjects

- Nothing to Report

Significant changes in use or care of vertebrate animals.

- Nothing to Report

Significant changes in use of biohazards and/or select agents

- Nothing to Report

PRODUCTS:

Publications, conference papers, and presentations

- Nothing to Report

Journal publications.

- Nothing to Report

Other publications, conference papers, and presentations.

- Nothing to Report

Website(s) or other Internet site(s)

- Nothing to Report

Technologies or techniques

-Nothing to Report

Inventions, patent applications, and/or licenses

-Nothing to Report

Other Products

-Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Kamal Khanna, Ph.D.
Project Role: Principal Investigator
Nearest Person month worked: 4
Funding Support: 30% of Dr. Kamal Khanna's salary support is provided by the SOM

Name: Jennifer Rutowski
Project Role: Research Assistant I
Nearest personal months: 12
Contribution to Project: Ms. Rutowski is the technician in the lab and she has assisted technically in virtually every experiment especially the ones dealing with flow cytometry and processing of tissues for imaging and animal husbandry.

Name: Stephen Yeung

Project Role: Graduate Student
Nearest person month worked: 4
Contribution to Project: Has performed all the experiments above with the help of Ms. Rutowski
Funding Source: Mr. Yeung was funded on this grant until he received a fellowship and was placed on a NIH T32 training grant, under which he is still funded.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

None

What other organizations were involved as partners?

Nothing to report

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Nothing to report

QUAD CHARTS: Nothing to report

APPENDICES

None