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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-□, 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.

I would like to note that because of my move from University of Connecticut to NYU we were delayed in initiating all the work outlined in the grant. The move and setting up the lab took longer than we had expected. Moreover, NYU has a strict requirement that all the mice that are imported to NYU facilities be rederived. This requirement further delayed the experiments because we had to rederive the PXR KO mice and the PXR floxed mice. The PXR floxed mice are now rederived and we have again begun to cross these mice to CD169-Cre and Villin-Cre. The crossing will take additional time to obtain PXR-floxed-homozygous/Cre-hets. However, we are now set to accomplish all the goals outlined in the grant.

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.

First, we imaged the colonic tissue from WT and PXR deficient mice. The colon was PLP fixed and frozen in OCT. 20um thick sections were cut and imaged using antibodies against CD169, CD11c, F480, EpCAM and CD11b. 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, the localization and number of CD11c+ cells which likely represents dendritic cells (DCs) in the colon of WT and PXR deficient mice appeared to be dissimilar. Majority of the CD11c cells were on the top of the villus structure in epithelial layer in WT and PXR KO mice, however, the number of CD11c+ cells was reduced in PXR KO mice. Furthermore, the DCs appeared to be in an organized network in WT mice, while in PXR KO mice the network structure was not as organized.

We also imaged the CD169+ macrophages in colon of WT and PXR deficient mice (Fig. 2). Interestingly, the number and concentration of CD169+ macrophages were dramatically increased in the colon of PXR KO mice when compared to WT mice. The networks of CD169+ macrophages in the PXR KO colonic tissue was more organized and the clusters of these cells were more pronounced in the KO mice compared to WT mice.

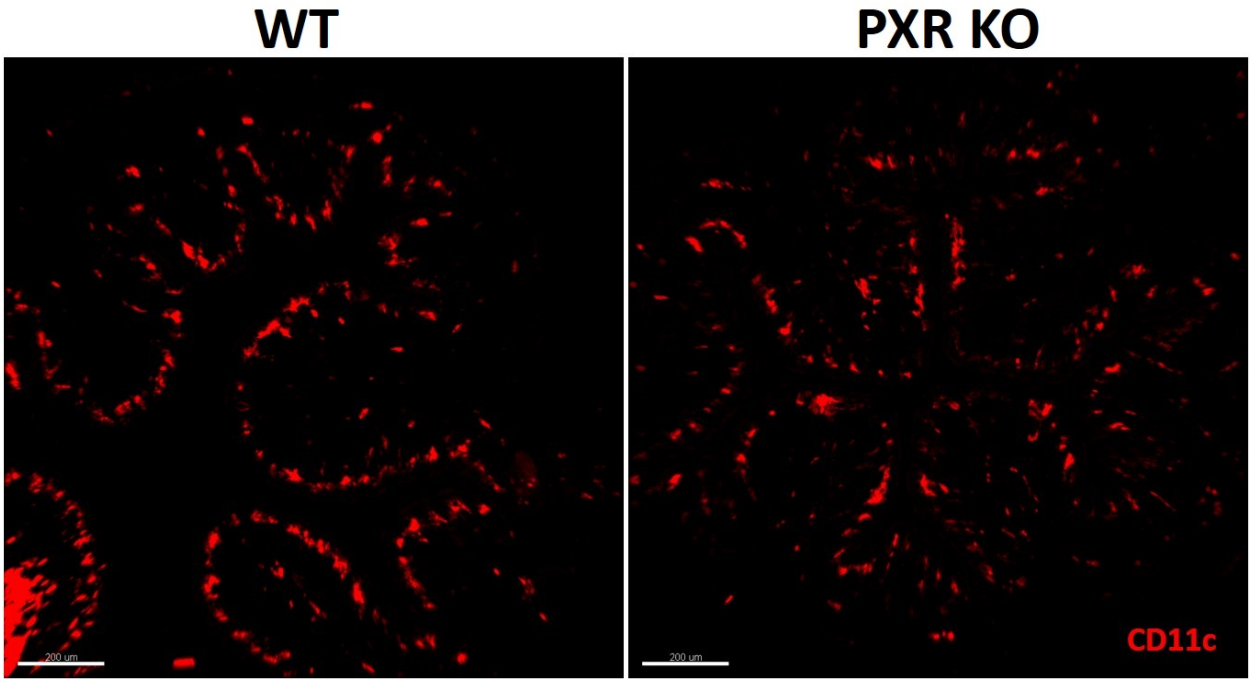

Fig.1. Colonic tissue from WT and PXR KO mice stained for CD11c.

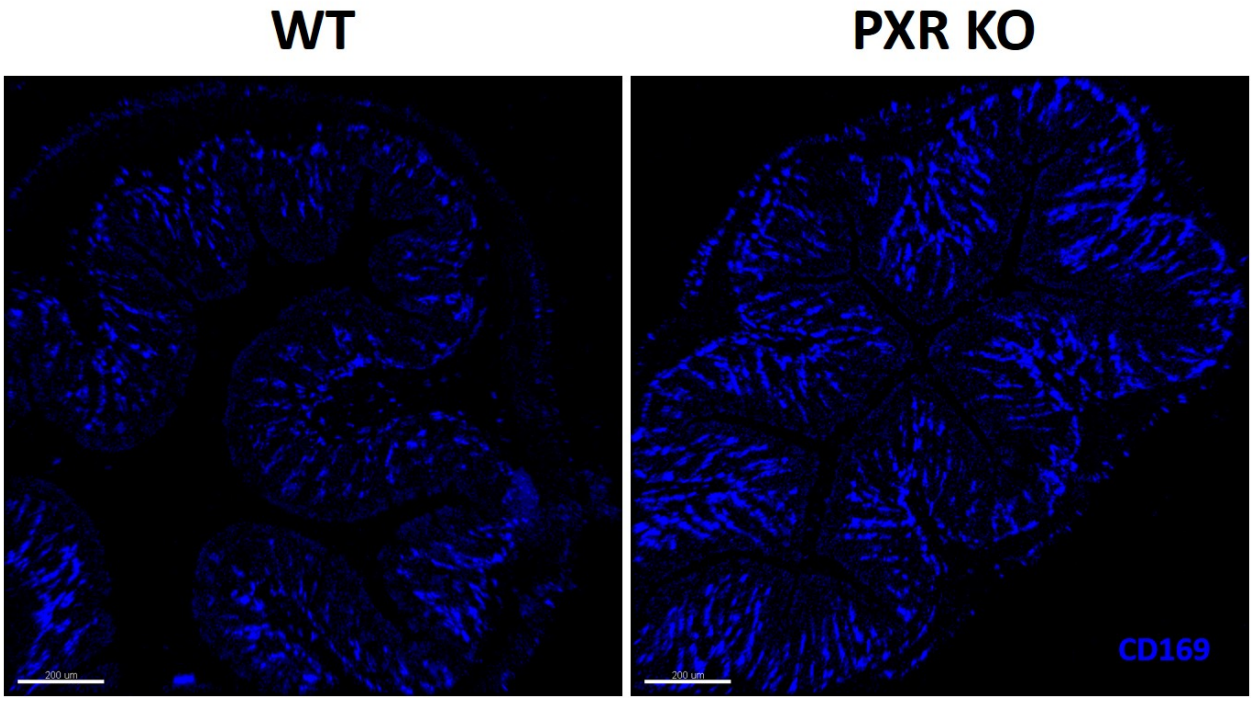

Fig.2. Colonic tissue from WT and PXR KO mice stained for CD169.

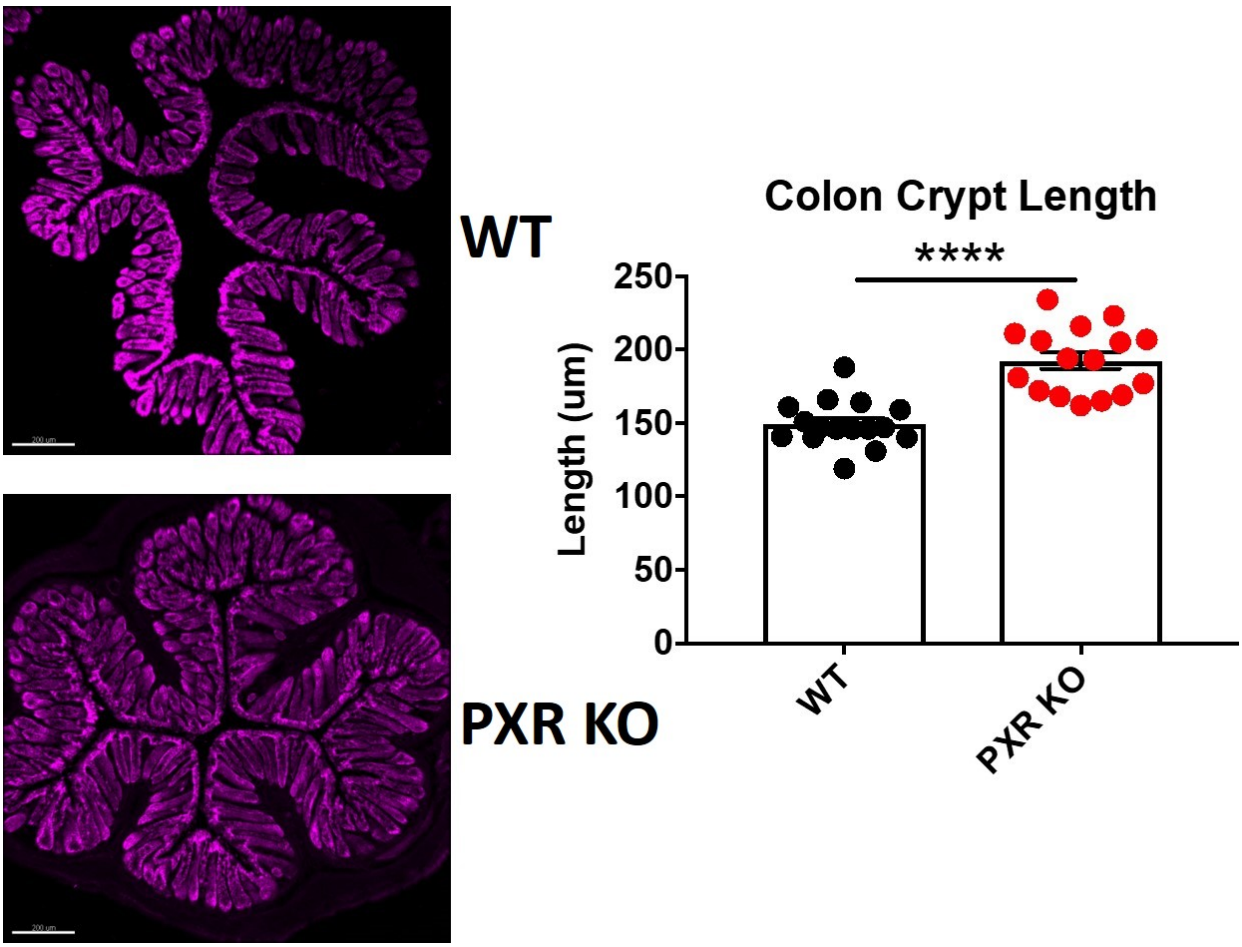

Fig.3. Colonic tissue from WT and PXR KO mice stained for EpCAM (purple) and the colonic crypt length was quantified using Imaris software. ****p<0.00005. Student t-test was performed on several different colonic tissue samples from 4 different mice.

The data with respect to the CD169+ macrophages in PXR KO mice suggested increased inflammation in PXR KO colons even at steady state. One important geographical result of elevated inflammation in the gut tissue is increase in colonic crypt length. Thus, we stained the colonic sections of WT and PXR KO mice with the epithelial marker EpCAM and used the Imaris software to quantify crypt length. Indeed, we observed a significant increase in crypt length in PXR KO mice.

Next we undertook an extensive flow cytometric analysis of myeloid cell infiltration in the colonic and small intestinal tissue of WT and PXR KO mice at steady state. 18 week old male mice in both groups were sacrificed and the colon and small intestines were enzymatically treated to acquire single cell suspension.

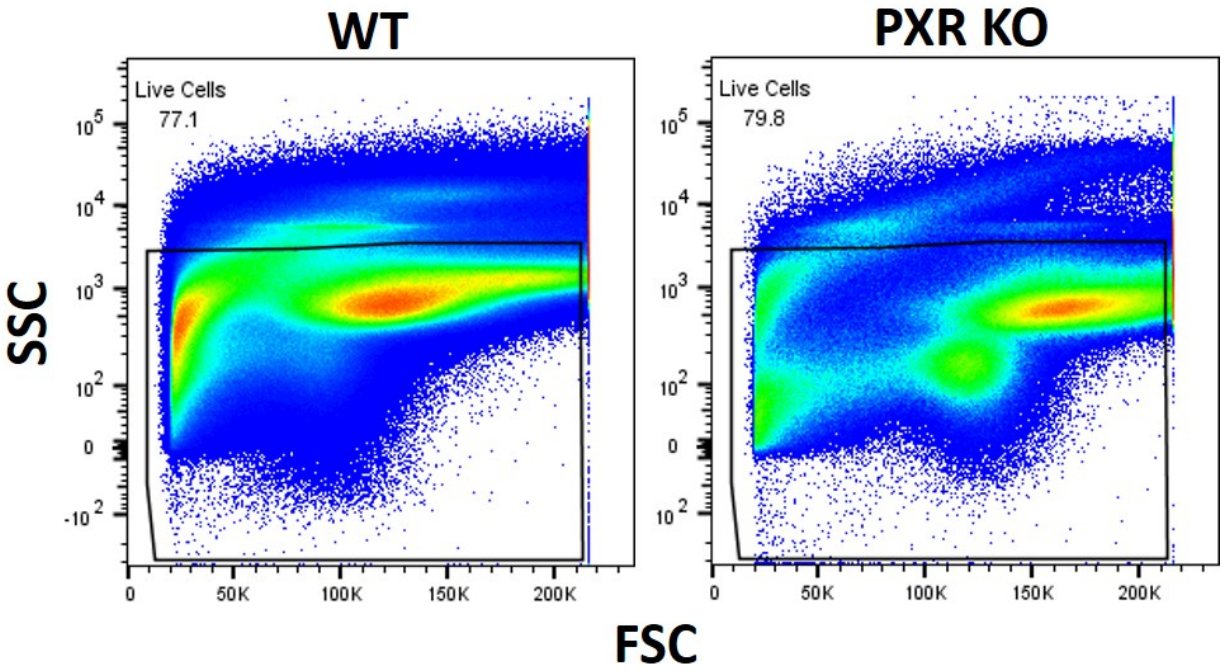

Fig.4. Forward and side scatter dot plot of a representative WT and PXR KO mouse. The cell population represents colon single cell suspension. Percoll gradient was used to enrich for hematopoietic cells. Representative data from 4 different mice are shown. Note the dramatic increase in FSC in cells isolated from PXR KO mice compared to WT mice.

As shown in Fig4, the increase in FSC (an indication of cell size) among the PXR KO colonic cells (compared to WT) again suggested an increased activation state of hematopoietic cells in PXR KO mice. It should be noted this increased inflammatory phenotype in PXR KO colons exists even though the mice were housed in pathogen free environment and thus, were under steady state conditions. Even though the data thus far clearly showed an increase in inflammation in PXR KO colons the percentage of live cells obtained from WT or PXR KO colonic tissues were equal (Fig.5)

Next we concentrated on determining if the imaging data could be validated using flow cytometric analysis. Indeed, the frequency of total number of CD169+ macrophages (that were also CD11b+ F480+) was significantly increased in the colons of PXR KO mice when compared to WT mice (Fig.6). Thus, in line with the imaging data, the frequency of CD169+ macrophages was also increased in PXR KO mice when compared to WT mice. Furthermore, among the CD169+ macrophages the subset that expressed the CX3CR1 chemokine receptor was particularly increased in PXR KO colons vs. WT colons (Fig.7)

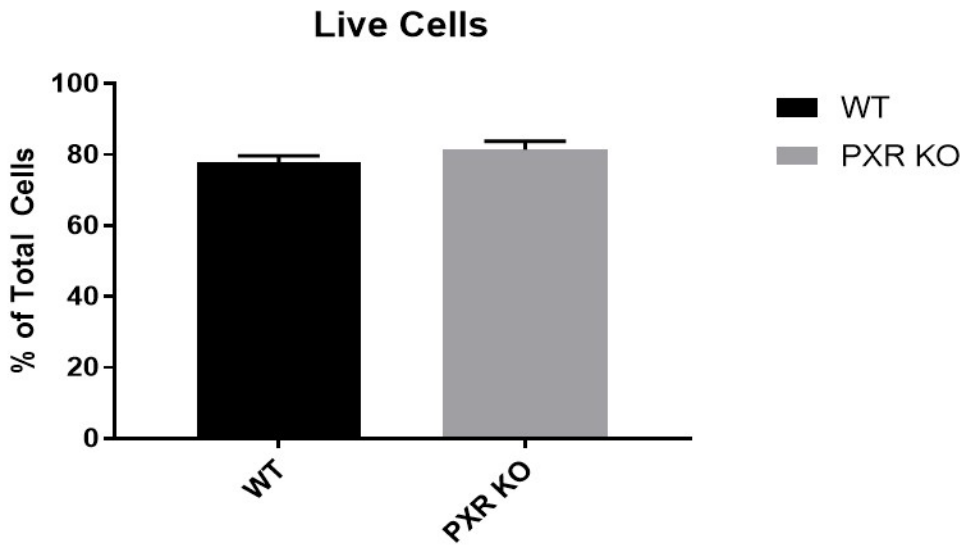

Fig. 5. Live cells recovered from the colon of each group of mice as indicated by live/dead stain

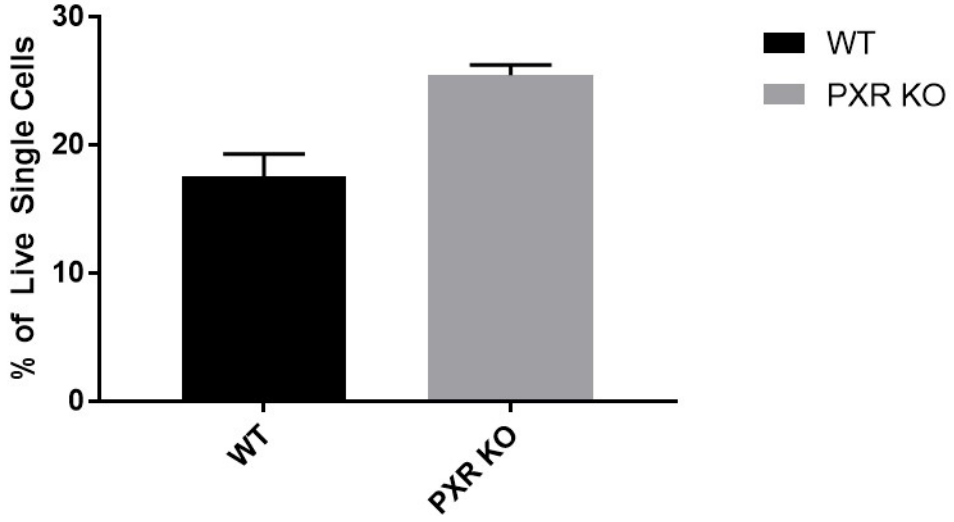

Fig.6. Frequency of CD169+ macrophages in the colon of the indicated strains of mice.

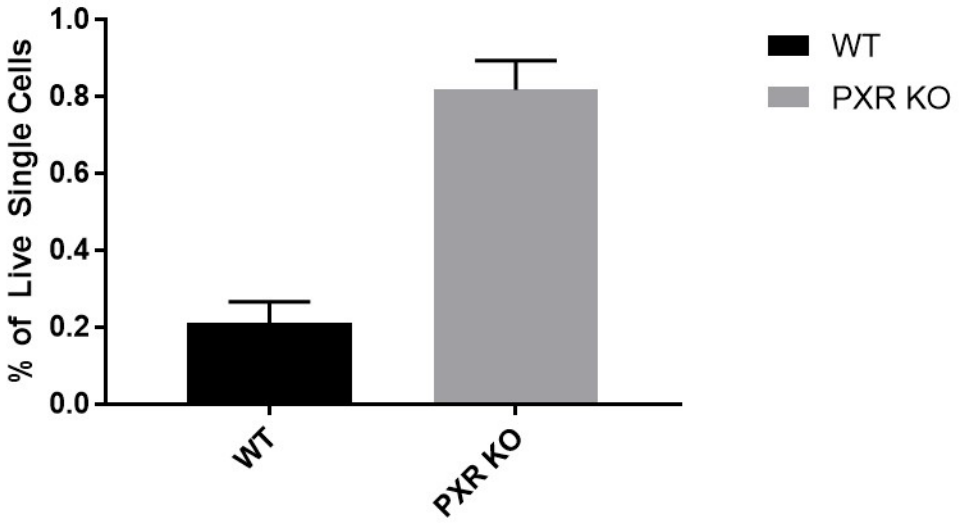

Fig.7. Frequency of CD169+ CX3CR1+ macrophages in the colon of the indicated strains of mice.

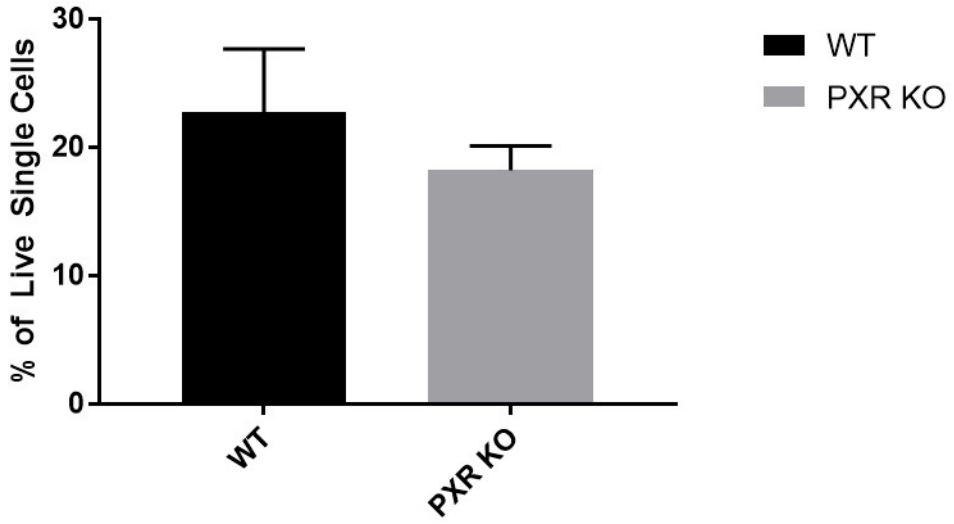

Fig.8. Frequency of CD11+ dendritic cells that fail to express any macrophage specific markers such as CD11b and F480 in the colon of the indicated strains of mice.

The flow cytometric data agreed with the imaging data where we observed a decrease in the number of CD11c+ DCs.

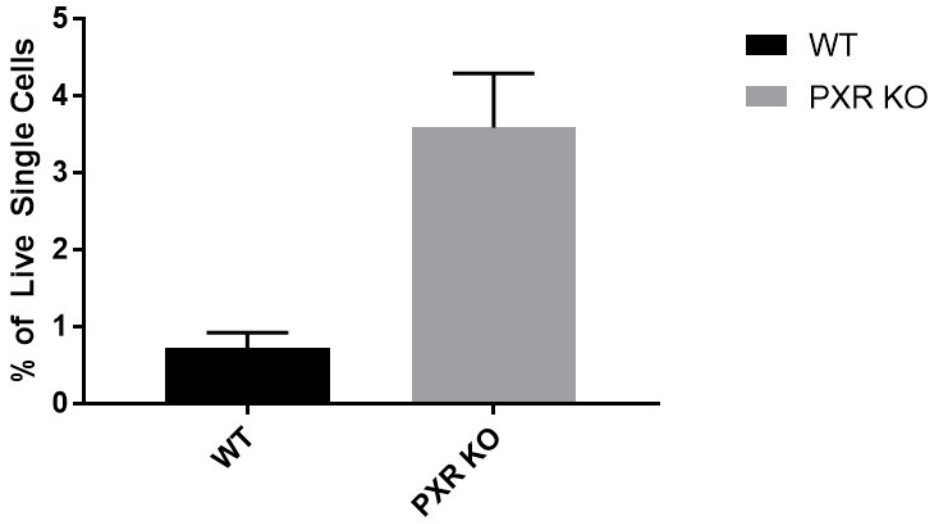

Fig.9. Frequency of LY6G+ neutrophils in the colon of the indicated strains of mice.

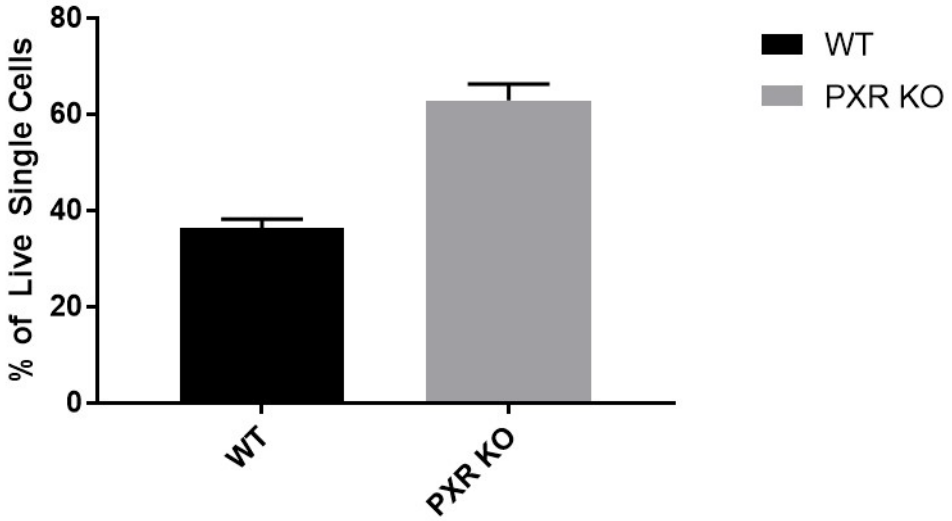

Fig.10. Frequency of LY6C+ monocytes in the colon of the indicated strains of mice.

Judging from our macrophage data, we had hypothesized that the increase in CD169+ macrophages may lead to increased recruitment of neutrophils and monocytes, and that is indeed what we observed. There was a striking increase in the number of neutrophils and monocytes (Fig.9 and 10 respectively) in the colon of PXR KO mice compared to WT mice. These data demonstrate a novel mechanism by which PXR may wield its immune-modulatory effects in the intestines. Thus, conditional ablation of PXR specifically in CD169+ macrophages by using PXR-floxed mice will be important and will likely yield interesting and mechanistic results.

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

- Jennifer Rutowski received training in imaging and flow cytometric analysis and mouse husbandry under this grant. She attended several training sessions for lab and animal handling safety.
- Graduate Student Stephen Yeung was partially funded by this grant before he received a fellowship and was put on a T31 training grant. He attended an intensive imaging and microscopy training program at NYU.

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.

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

It should be noted that because of my move from University of Connecticut to NYU we were delayed in initiating all the work outlined in the grant. The move and setting up the lab took longer than we had expected. Moreover, NYU has a strict requirement that all the mice that are imported to NYU facilities be rederived. This requirement further delayed the experiments because we had to rederive the PXR KO mice and the PXR floxed mice. The PXR floxed mice are now rederived and we have again begun to cross these mice to CD169-Cre and Villin-Cre. The crossing will take additional time to obtain PXR-floxed-homozygous/Cre-hets. However, we are now set to accomplish all the goals outlined in the grant. Thus, all the aforementioned mice will be crossed and the models will be used to complete the next important tasks: Characterize the epithelial and macrophage intrinsic mechanisms by which PXR regulates homeostasis in the gut mucosa after enteric infection.

These data demonstrate a novel mechanism by which PXR may wield its immune-modulatory effects in the intestines. Thus, conditional ablation of PXR specifically in CD169+ macrophages by using PXR-floxed mice will be important and will likely yield interesting and mechanistic results.

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.

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

- I would like to note that because of my move from University of Connecticut to NYU we were delayed in initiating all the work outlined in the grant. The move and setting up the lab took longer than we had expected. Moreover, NYU has a strict requirement that all the mice that are imported to NYU facilities be rederived. This requirement further delayed the experiments because we had to rederive the PXR KO mice and the PXR floxed mice. The PXR floxed mice are now rederived and we have again begun to cross these mice to CD169-Cre and Villin-Cre. The crossing will take additional time to obtain PXR-floxed-homozygous/Cre-hets. However, we are now set to accomplish all the goals outlined in the grant.

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 F31 training grant.

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

The PI received a RO1 from NIH:

Grant Number: 1 R01 AI143861-01

Principal Investigator: Khanna, Kamal Mohan

Project Title: Novel lung resident interstitial macrophage subset with distinct localization and polarization

Institution: NEW YORK UNIVERSITY SCHOOL OF MEDICINE

Award Issue Date: 12/28/2018

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