To investigate the impact of gut bacteria on efflux transporter expression and function in gastrointestinal mucosae

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ABSTRACT

Gut microbiome diversity may alter oral bioavailability and contribute to individual variations in drug therapy. Therefore, understanding molecular links between the gut microbiome and oral drug disposition is critical for realizing the goals of precision medicine. The purpose of the study was to characterize the function of P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) using intestinal organoids derived from germ free and humanized mice. Typically, transport across the epithelial cell layers occurs by active transport and passive diffusion. Using mouse models that differ in bacterial content and fluorescence scanning, and live imaging techniques we studied the impact of bacteria on protein expression and function. The accumulation of membrane permeable acetoxymethyl calcein (calcein-AM), a widely used probe to monitor P-gp mediated efflux activity, in organoids was determined with and without the presence of P-gp inhibitors Cyclosporine A and LY335979. The uptake of calcein-AM was higher within the enterocytes of humanized mice colonoids compared to those obtained from germ free mice. These results correlated with immunohistochemistry on tissue explants that showed a lower expression of P-gp in germ free colon compared to the humanized mice colon. Both P-gp inhibitors showed an increase in calcein-AM uptake owing to the reduced transepithelial transport. Germ free colonoids showed a higher uptake of the BCRP probe Pheophorbide-A (Phe A) within the enterocytes as compared to humanized mice colonoids. These results correlated with Immunohistochemistry experiments that showed a lower expression of BCRP in germ free colon compared to humanized mice colon. These results demonstrate that intestinal organoids can be used as a powerful model to study expression and function of efflux transporters in the GI tract.

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INTRODUCTION

Most drugs available today are administered via the oral route. Hence, it is important to understand the impact of gut milieu on oral bioavailability of drugs as it potentially affects their therapeutic activity. Oral drug bioavailability is affected by physicochemical factors such as solubility, lipophilicity, and hydrogen bonding ability of drug molecules, as well as biological factors including the expression of transporters and drug metabolizing enzymes.[1] Recent evidence points to the role of gut microbiota on the absorption and disposition of orally administered drugs by altering the expression of drug metabolizing enzymes and transporters.[1,2]

Upon oral administration, drugs are absorbed into the systemic circulation primarily by passive diffusion or active transport. The passive diffusion of lipophilic drugs mostly occurs by the transcellular route, whereas the low molecular weight hydrophilic compounds are likely absorbed across the paracellular spaces between the epithelial cells. The influx or efflux of endogenous substances and xenobiotics is mainly governed by transporters that are expressed in the enterocytes.[3,4,5] Membrane transporters located on the apical membrane of intestinal epithelial cells, such as permeability-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), restrict the transcellular permeability of many drugs and limit their oral absorption by reducing their uptake into the enterocytes and facilitating their transport back into the lumen.[1,2,6]

The advent of high throughput genomic technologies and a major shift in healthcare towards precision medicine has enhanced our ability and necessity to understand various environmental factors that affects drug disposition. One such factor is the relationship that exists between the host cells and the gut microbiome.[7,8] Therefore, understanding this host-

microbial cross talk and its effect on oral drug disposition is critical for realizing the goals of precision medicine.

The gut microbiome, which is the largest and the most diverse of the human microbiomes, represents an ecological community of over a trillion commensal, symbiotic and pathogenic microbes residing in the gastrointestinal (GI) tract.[9] In addition to playing an important role in extracting critical nutrients from the ingested food, gut microbes affect drug disposition.[10] The likelihood of observing high interindividual variability between the host cells and gut microbiota makes this research area highly interesting to understand and accurately predict a drugs pharmacokinetics for a given population.[11] In one study by Franzosa et al., 2015, they observed that microbial communities are quite stable over time and are unique to a host, suggesting that microbial "fingerprints" may serve as unique study models in the future.[12]

Furthermore, the microbial composition varies along the GI tract. It exists in most of the GIT but isn't seen in the stomach and upper small intestine due to the acidic conditions in these parts from the gastric content. Therefore, a large number of bacterial microflora fills the human's distal small and large intestines.[13] Drugs that suffer from low solubility and/or low permeability especially extended–release formulations reach the lower part of the intestinal tract and their absorption is more susceptible to the potential effects intestinal microflora have on the GI epithelium. [10,14]

The impact of the gut microbiome on the pharmacokinetics of drugs and xenobiotics is well known to us with at least 50 drugs known to be metabolized by bacteria. [8,11,15] For drugs that affect the composition of microbiota, drug-drug interactions that may arise could simultaneously affect the bacterial biotransformation necessary for a bacterial

substrate.[16] Additionally, bacterial metabolites i.e. metabolites produced by bacteria are known to mediate drug-drug interactions. An example of this is the Japanese marketed drug Sorivudine, an antiviral drug that was withdrawn due to the interaction between a gut metabolite of Sorivudine and anticancer drug 5-fluorouracil that caused extreme toxicity due to the buildup of fluoropyrimidine metabolites.[8,16,17]

Subsequently, gut microbiota may express enzymes that affect drug metabolic activity by either activating or deactivating them. Some of the known metabolic reactions that impact the biological activity of drugs are hydrolysis, dihydroxylation, acetylation, deacetylation, proteolysis, deconjugation, and deglycosylation processes.[8,14] Among these reactions, hydrolysis of the glucuronide conjugates is the most important metabolic reaction that is mediated by the glucuronidase enzyme and produced by the bacterial microflora found in the GI Tract of humans.[2] Hydrolysis of glycosidic linkage is one of the best-known examples of bacterial enzyme activity as well. The principal glycosidase are β-glucosidase and β-glucuronidase release the parent compound by hydrolyzing the glycosidic bond of glycoside and glucuronide conjugates, respectively, to release the parent compound.[2]

Additionally, our diet plays an important role in the way our microbiome metabolizes food. Diets rich in carbohydrates contribute to the production of short-chain fatty acids (SCFAs), which are largely thought to be beneficial to health. Carbohydrates are an essential energy source and their fermentation by anaerobic bacteria produces mainly acetic, propionic, and butyric acids, which affect colonic epithelial transport, colonic metabolism, provide energy for heart, muscle, brain and kidney. High colonic

butyrate concentrations enhance gut motility and limit the growth of pathogenic microorganisms.[16,18]

Further, gut microbiota produced substances have shown to affect organs such as the brain as well at relatively low concentrations. In addition to SCFAs, the gut microbiota produces a number of neurotransmitters such as serotonin, dopamine and noradrenaline that can directly affect our mood, sleep, memory and appetite. SCFA's have also been shown to induce an 8- to 10-fold increase in serotonin release in an in vitro colonic mucosal system.[19] Tryptophan, an α-amino acid that is used in the biosynthesis of proteins is converted to 5-hydroxytryptamine by gut microbes. A diverse number of metabolites generated by the gut microbiota can impact production of these neurotransmitters and metabolites, and in turn participate in the gut-brain axis to form the microbiota-gut-brain axis. [19,20,21]

The synergistic effect of efflux transporters and enzymes in the enterocytes also plays an important role in drug metabolism. Since, most compounds which are substrates for P-gp are also substrates for CYP3A4 and are both expressed in the enterocytes this makes it possible for both CYP3A4 and P-gp to act synergistically. However, the extent to which they affect an individual protein depends on their spatial relationship and the drug's physicochemical properties causing them to be a substrate, inducer or inhibitor.[22] Nuclear receptors, particularly, the CAR and PXR regulate the expression levels of these enzymes and transporters.[23] Claus et al., 2011 conducted a study to analyze the impact of the microbiota on hepatic gene expression levels of drug-metabolizing enzymes using germ free and conventionally raised mice. The expression levels of Cyp2c29, Cyp3a11, and Cyp8b1 were significantly lower in the germ-free mice. However, after 20 days of bacterial

colonization their levels were no longer reduced and an increase in Cyp2d9 and Cyp2e1 were observed in the germ-free mice compared with the conventionally raised mice.[24]

However, there are several challenges in evaluating the involvement of gut bacteria in drug metabolism. Some of the methods deployed to study the impact of gut microbes on drug metabolism includes incubating drugs in vitro with intestinal content, using fecal samples to isolate microbes, or screening representative strain libraries but several problems have been observed with these methods.[8,25]

For example, gut microbial presence varies along the length of the gut and thus fecal samples may not provide an accurate representation of microbial content.[26] In vivo evaluation of gut bacterial metabolism in animals is feasible but limitations of large interspecies differences in microbiota composition and distribution makes it difficult to study microbial effects on metabolism.[14] Drugs are subjected to animal experimentation before entering human clinical trials as it provides a good basis to understand the risks involved. However, the scientific community is trying to develop newer methods to move away from this practice despite its advantages due to the extensive use of animals for these studies.[5] In an attempt to reduce these practices, the use of intestinal cell models that are less expensive and more convenient to better understand the bioavailability is being incorporated. Not only do they provide a good basis to understand drug pharmacokinetics but also demonstrate the relationship between the host, pathogens and intestinal microflora and their potential as in vitro platforms for preclinical research is well established.

The discovery of organoid technology, that provides near physiological attributes has improved our understanding of stem cells and has provided the research community with a powerful tool to model human physiology and disease. In our current study, we have

attempted to study the use of organoids as a model to understand the effect of gut microbes on efflux transporter expression and function, and in turn provide an understanding of the function of the enteroendocrine system in drug metabolism.

Historically, it has been increasingly difficult to develop in vitro models that faithfully mimic gut physiology primarily because they're modelled using cell lines and explanted tissues, both of which have limitations.[27] Although 2-D structures are easy to achieve and relatively inexpensive, errors and inconclusive results are unavoidable because only a certain section of the specimen is from the bulk of the tissue and thus lacks the integrative physiology. On the other hand, tissue explant cultures recreate the characteristic 3-D architecture and cellular heterogeneity and offer the opportunity to investigate physiologically relevant primary cells.[28] However, their use is limited by their inability to survive too long, the need for multiple tissue donors and the lack of direct genetic manipulation. The recent development of in vitro organoid culture systems offers the opportunity to address these limitations. One of the methodologies for gut organoid culture is the use of tissue stem cells. Results achieved through their manipulation are being used to answer fundamental questions of gastrointestinal physiology.[29]

Organoids derived from resident adult tissue stem cells

One of the major challenges of tissue explant cultures is their inability to survive long-term growth. Clevers and colleagues established a culture system which allowed the long-term growth and expansion of intestinal epithelia from either purified intestinal crypts or single Lgr5+ stem cells by identifying critical components of the intestinal stem cell niche, often

called mini-guts or enteroids.[30,31] Enteroids are grown in 3D, in laminin-rich Matrigel, with a defined set of niche factors including epidermal growth factor (EGF), noggin and R-spondin and have the ability to form three-dimensional (3D) intestinal organoids by self-organizing into crypt–villus organoids even in the absence of a non-epithelial niche.[29] An important factor that induces the proliferation is the interaction of the R-spondin-1, a Wnt signal enhancer and the Lgr5. R-spondin-1 causes hyper plasticity of the crypts which means that it promotes cell proliferation and helps in the growth of the tissue or organ. One of the important functions is that it provides the architecture of structural proteins such as collagen, laminin and fibronectin that mimic the cellular environment. Integrin's located on the cell surface bind to these proteins and activate a series of cellular responses. Thus, in order to achieve tissue homeostasis and be able to mimic that in vitro it is imperative that we develop models that not only replicate the mechanical properties but also the chemical properties at all stages of cell growth.[29,30] Within days, crypts or single stem cells form cystic single-cell epithelial structures with a central lumen and have a similar morphology as seen in vivo. The orientation of the basolateral and luminal sides is such that the basal side faces the outside towards the matrigel whereas the enterocytes occupies the luminal side. These crypt-like budding structures ultimately organize into multiple discrete budding crypts, harboring intercalated stem and Paneth cells at their base and associated villus-like regions comprising the various differentiated cell lineages, absorptive enterocytes, secretory goblet and EECs.[30,32] These cultures are grown and passaged (every 5-7 days) and exhibit normal polarization, where the apical membrane has microvilli and faces the central lumen, whilst the basolateral side faces outward.[33] Organoids can be generated from any segment of the gut; duodenum, jejunum, ileum and colon. The high

production of Wnt3A required for stem cell self-renewal by their resident Paneth cells allows small intestinal enteroids to grow in minimally effective media described above and retain their regional identity in culture.[29] In the presence of Wnt3A these organoids more closely resemble the cells of the crypt and are therefore in a relatively non-differentiated state. Removal of Wnt3A inhibits stem cell proliferation, allowing a more differentiated state to be achieved.[29] The main advantages of using adult stem cell-derived enteroids include segment specification, ease of genetic manipulation, simplicity and speed of generation

Considerations for organoid use

There are several general shortcomings for intestinal organoids. These models are able to mimic the structural functionality but do not necessarily mimic the in vivo tissue conditions, they lack vasculature, lymphatic system and functional adaptive and innate immune systems and hence do not provide a holistic resemblance to in vivo conditions.[34] Other general disadvantages include inconsistencies within each culture and access to the luminal side of the epithelium. Organoid 3D architecture is not regular, the crypt villus structures are variable in size and shape from one organoid to another, as is the degree of differentiation. Luminal access is restricted and requires direct injection of agents into the luminal side of each individual organoid, this is time consuming and technically challenging.[35] Luminal access can be achieved by fragmenting organoids and growing them on semipermeable supports enabling controlled access to both apical and basolateral surfaces. However, 3-D organoids offer a good comparison for short term conditions but for a long-term study only in vivo studies seem progressive. Overall, these models enable

us to bridge the gap between simple cell models and animals and can be used as effective experimental tools.[36]

Models used to grow Organoids

There are several options for using and generating genetically manipulated organoids. The simplest being the culture of organoids from transgenic and knockout mice which does not require the maintenance of a colony, only the transfer of donor tissue or frozen organoid cultures between labs, making primary genetically altered material very accessible. Reagents can also be applied directly into the culture medium and/or Matrigel to achieve targeted gene knockdown. This is in contrast to the techniques which require dispersing organoids or manipulation of iPSCs before differentiation into organoids. Thus, it can be understood that several mouse models serve as fascinating and impactful means to conduct research. The mouse models described below were used in our current study to understand the impact of bacterial differences on protein expression.[37]

Germ Free and Humanized mouse models

The human gut is a diverse ecosystem that harbors10^{13}-10^{14} microorganisms and hence its role in ensuring proper functioning within the gut is paramount.[38] Owing to their large numbers these species interact with the host extensively both physiologically and pathologically. For example, their role in the development of the mucosal and systemic immune system and the renewal of the intestinal epithelium.[10] In humans they play a key role in vital processes like vitamin biosynthesis, fermentation of carbohydrates to list a few.

However, the role of microbial signaling on their host to cause a physiological response hasn't been well understood as yet.[39] Studies have shown the role of gut bacteria in maintaining intestinal barrier function. To study the role of microbes on host responses, the germ-free mouse model works well and has been used extensively in the past to study the effect of bacteria when compared with conventional mice and humanized mice. Germ free mice are bred in a sterile environment or a gnotobiotic facility where they have no exposure to bacteria or germs. Even the food and water provided is heated at temperatures over 100 °C before being delivered to its cage through a sterile, fail-safe system of double doors. A germ free colony is started by ensuring that the young animal is carefully removed from the mother's womb to start a germ-free colony, one must remove a young animal from its mother's womb and raised in a sterile cage and only exposed to food, water, and other equipment that has also been sterilized. Once the colony has been created, the germ-free mother can give birth to new animals naturally that are also germ-free. These germ-free mice have been seen to have altered immune systems, hearts, lungs, lymph nodes, metabolisms, and even brain development and behavior. However, when these mice are colonized with a mixture of bacteria or humanized from humans or other animal donors, the results are reverted to normal or comparable to conventional mice.[40] These "humanized" gut microbiomes have provided us with significant information regarding the changes in the gut environment and difference in its effects compared to germ free mice. Analyses on these mice has revealed that key microbial activities were successfully transferred from the donor to the recipient animal eg: the conversion of cholesterol to equol, effect of diet on microbial gene expression and the presence of microbes across the length of the GIT compared to what is known.[41] However, interspecies differences exist as the gut

environment of mice varies from humans limiting the direct extrapolation of results and hence we colonize germ free mice with human fecal matter or other donors. These models have proved to be effective in the past and serve as good markers to test microbial differences and their effects on host physiology.

In our present study, we are using Lgr5+-CBC stem cells that form long-term cultures (> 1.5 years) that self-organize in the absence of a non-epithelial niche to form three-dimensional (3D) intestinal organoids.[29] Organoids grown in-vitro recapitulate critical physiological features of the GI epithelium.[32,42,43] Moreover, organoids serve as efficient experimental, diagnostic and therapeutic tools as they are amenable to techniques such as Immunohistochemistry and confocal immunofluorescence.[29] In this study, we employed organoids to investigate the influence of gut microbiota on the expression and function of major efflux transporters that influence oral drug absorption

MATERIALS AND METHODS

2.1 ANIMALS

Eight-to-twelve week old Swiss Webster mice employed in the current study include: conventionally raised (CR) mice with normal mouse microbiota from birth; germ-free mice raised in the aseptically maintained isolators; and humanized mice-germ free mice that received fecal microbiota from a healthy human donor. All mice were bred and raised in the germ-free mouse core at the Mayo Clinic in accordance with National Institutes of Health Guide for the Care and Use of Laboratory animals and by following the protocols approved by Mayo Clinic Institutional Animal Care and Use Committee.

The mice that are referred to as "humanized" are germ-free mice that harbor human microbiota. These were generated by administering a gavage of 300 µl of a 1:1 suspension of pre-reduced PBS, which was kept in an anaerobic cabinet overnight to expel dissolved oxygen, and fecal sample obtained from a healthy donor to mice at 4 weeks of age. To ensure that the results obtained from humanized mouse tissue are not unique to a single human fecal microbiota, two independent human donors were employed.

2.2 PREPARATION OF ORGANOIDS

Mouse tissue collection

In these studies, distal small intestine and proximal colonic segments of the gastrointestinal tract obtained from germ free, conventional and humanized mice were used. The tissues were resected immediately after sacrificing the animal and were placed in ice-cold Kreb's Ringer bicarbonates buffer (7.03g NaCl, 0.44g KCl, 1.30g $NaHCO_3$, 0.19g NaH_2PO_4, 2.07g Mannitol, 45.8mM $CaCL_2$, 25.7mM $MgCL_2$) bubbled with carbogen (97% oxygen,

3% carbon dioxide), and stored on ice until further processing. Then the underlying muscle layers were removed with surgical scissors and the tissue was chopped into 2-mm pieces for harvesting intestinal crypts.

Isolation of mouse intestinal crypts

The procedure to obtain crypts from mouse small intestine and colon was adapted from previously published work.[1] The mouse colon intestinal sections were washed with ice-cold PBS in a 50-mL conical tube at least 15 times until the supernatant was clear. The tissue sections were added to 25 mL of Gentle Cell Dissociation Reagent (StemCell technologies, Vancouver, Canada) and incubated by continuous shaking on a rocking platform (75 rpm) for 15 minutes at 32 ^{o}C. Then the tissue sections were allowed to settle to the bottom of the conical tube and the supernatant containing fecal remnants was removed. Subsequently, the tissue sections were suspended in 10 mL of cold PBS containing 0.1% bovine serum albumin (BSA) and gently pipetted back and forth 10 times using a 10 mL pre-wetted pipette. The tissue sections were allowed to settle under gravity, and the supernatant was filtered through a 70 μm filter into a 50-mL conical tube and labeled as Fraction 1. This procedure was repeated three times to generate the subsequent fractions. The supernatants containing crypts were transferred to 15 mL tubes. These sections were then finally centrifuged at 290×g for 5 minutes at 4 ^{o}C to pellet the intestinal crypts.

Intestinal Organoid Culture

The isolated intestinal crypts were suspended in 10 mL of cold DMEM/F12 media in 15 mL tubes. The tubes were then centrifuged at 200×g for 5 minutes at 4 ºC, and the supernatant was discarded; the pellets were suspended in 150 µL of complete IntestiCult™ Organoid Growth Medium (StemCell technologies, Vancouver, Canada) at room temperature, and the tube was placed on ice. Then, 150 µL of undiluted Corning Matrigel® Matrix (150 µL) was added to this suspension and pipetted up and down to thoroughly suspend the pellet. An 80 µL aliquot of this suspension was pipetted to the center of each well on a pre-warmed 24-well plate to form a dome like structure holding the crypts and the plates were incubated at 37 ºC for 10 min to solidify the Matrigel. Then 500 µL of the IntestiCult™ Organoid Growth Medium (StemCell technologies, Vancouver, Canada) supplemented with 100 µg/mL of penicillin/streptomycin was added to each well without disturbing the dome. Lastly, PBS was added to any unused wells, and the plates were incubated at 37 ºC under 5% CO_2. The IntestiCult™ Organoid Growth Medium culture medium was changed every 3-4 days, depending on the growth of the organoids, by carefully aspirating the existing liquid medium and replacing with 500 µL fresh, room temperature IntestiCult™ Organoid Growth Medium.

Passaging mouse intestinal organoids

The Organoids were passaged after 7 days of the initial culture. Briefly, a 1 mL aliquot of Gentle Cell Dissociation Reagent was added to each well at room temperature. The dome of the organoids was dispersed by pipetting up and down multiple times, and the contents were transferred to a 15 mL conical tube. The tubes were incubated at 32 ºC on a rocking platform at 72 rpm. Then the tubes were centrifuged at 290×g for 5 minutes at 4 ºC, the

supernatant was discarded. The dispersed organoids were suspended in DMEM/F12 medium and centrifuged at 290×g for 5 minutes at 4 °C. The pellets were resuspended in 150 µL of complete IntestiCult™ Organoid Growth Medium and the organoid domes were plated onto the coverslip bottomed dishes as described previously.

2.3 CALCEIN-AM AND PHEOPHORBIDE- A EFFLUX AND CELL VIABILITY STUDIES

Calcein-AM, a P-gp substrate and Pheophorbide A, a BCRP substrate are widely used and marketed as markers for P-gp and BCRP mediated efflux activity. The effect of various P-gp and BCRP inhibitors on the accumulation of these fluorescent substrates in the organoids was examined.

After the first passage, the organoid dome was grown on coverslip bottom dishes for 3 days. The control dishes were incubated in media containing calcein-AM (0.5 µM) for 60 min at 37 °C or Pheophorbide A (15 µM) for 30 min at 37 °C. Alternatively, the organoids were preincubated in the presence of P-gp (Cyclosporin A, 5 µM or LY335979, 1 µM) or BCRP (Novobiocin, 15 µM) inhibitors for 30 min at 37 °C before adding the fluorescent substrates. Then the organoids were imaged live at 37°C under 5% CO_2 using the following microscopes:

a) Nikon TE2000-S inverted microscope (Chiyoda-ku, Tokyo 100-8331, Japan) equipped with Nikon FITC HQ and appropriate filters. Nikon's NIS elements AR 3.0 software was used to capture the images.

b) Zeiss Axio Observer Z1 spinning disk laser confocal microscope equipped with Photometrics Quantem 512 C EMCCD camera. The images of the control and treated

organoids were acquired at the same instrument settings and processed using Zeiss Zen software.

2.4 IMMUNOHISTOCHEMISTRY

The distal small intestinal and colonic tissue segments were collected from germ-free, humanized and conventional mice. The tissues were fixed with 4% paraformaldehyde in 1X Dulbecco's Phosphate Buffer Saline (DPBS) (Corning, Manassas, VA) at 4 °C for 4 h. Subsequently, they were sequentially transferred to increasing concentrations of 10%, 20% and 30% sucrose solutions in 1X PBS at 4 °C over three days. Then the tissues were embedded in Tissue-Tek® O.C.T. Compound (Sakura® Finetek) and properly chilled in 2-methyl butane maintained at -70 to -80 °C until the bubbling stopped. The embedded tissues were sectioned into 8 μm slices on a cryostat (Leica Biosystems CM3050 S Research Cryostat) and collected on Fisherbrand Superfrost Plus Microscope Slides. The sections were incubated with 3% hydrogen peroxide to quench any endogenous peroxidase and then washed with distilled water. The sections were blocked with normal goat serum (1:10 with 0.02 M PBS) for 20 min at room temperature. Then the blocking solution was discarded and the sections were incubated at 4 °C overnight with P-gp antibody [anti-rabbit IgG, RP1034 (Boster Biotechnology, Pleasanton, CA)] at 1:200 dilution, and or BCRP antibody [anti-rabbit IgG, PB9364 (Boster Biotechnology, Pleasanton, CA)] at 1:200 dilution. The tissue sections were washed three times for 2 min each with PBS and subsequently stained to detect P-gp or BCRP expression. The P-gp expression was detected by Cy-3 conjugated anti rabbit IgG secondary antibody kit (SABC kit SA1074, Boster Biotechnology, Pleasanton, CA). The tissue sections were incubated with 5 μg/ml (1:200 with 0.02 M

PBS)biotinylated Goat-Anti rabbit IgG secondary antibody in the kit at 37 ºC for 30 min , followed by incubation with 5 µg/ml of Cy-3 conjugated streptavidin (SABC-Cy3) (1:200 with 0.02M PBS) at 37 ºC for 30 min in the dark. For BCRP immunostaining, we used the Goat Anti-rabbit IgG secondary antibody, 5 µg/ml Cy-3 conjugate BA1032 (Boster Biotechnology, Pleasanton, CA) (1:200 with 0.02 M PBS) incubated at 37 ºC for 30 min in the dark. After the secondary antibody incubation, we washed the tissues three times for 2 min each time with 0.02 M PBS. For nuclear staining, the tissue sections were incubated with DAPI (1:5000 dilution with 0.02 M PBS) at 1 µg/ml for 15 minutes at room temperature followed by washing three times for 5 minutes each time with PBS. The immunofluorescence was detected with a Leica inverted fluorescence microscope (Leica microsystems (Buffalo Grove, IL), and captured using the Leica Application Suite X (LASX) software. The ImageJ software was used to analyze and quantify the immunohistochemical staining results.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed using the graph pad prism software package. The Student t-test was used to examine differences between two groups, while one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test;was used to determine differences among multiple groups. A p-value of <0.05 was considered statistically significant.

RESULTS

Culturing intestinal organoids: The organoids were cultured from intestinal segments of various mice, and we assessed the morphology of the colonoids and observed their growth over the duration of the culture. They grew showing round cystic structures on Day 3 after the first passage, and then formed asymmetric structures with crypt-like protrusions on Day 5. We then studied the expression of P-gp and BCRP in the colon of germ free and humanized mice in tissue explants and three-dimensionally cultured organoids. The functional aspect of these transporters was studied in the organoids. We then correlated differences in the cellular accumulation of the fluorescent probes with the expression of respective transporters in the mucosal explants of these mouse models with and without human microbiota.

3.1 EFFECTS OF VARIOUS INHIBITORS ON CALCEIN-AM ACCUMULATION IN COLONOIDS

The effect of P-gp inhibitors, Cyclosporine A and LY335979, on the accumulation of calcein-AM in colonoids was studied. The intense fluorescent signals of free calcein were observed real-time by confocal microscopy in the organoids of both germ free and humanized mice colonoids 1h after the incubation with a slight contrast against those in the outer space of organoids (Fig. 1a and Fig. 2a). This clearly shows that the colonoids in the culture were able to conduct active transepithelial transport of calcein-AM in the basal to apical direction, and this could be attributed to the involvement of P-glycoprotein.

The specific and potent P-glycoprotein inhibitor LY335979 showed an increase in the accumulation of calcein-AM over the control in both germ free and humanized mice colonoids (Fig 1c and 2c). The efflux of calcein-AM was suppressed in the presence of

LY335979 at 1 μM and can be seen in the higher fluorescent intensity of the area outside of the lumen, indicating the enhanced uptake of free calcein-AM into the cells. Treatment with Cyclosporine A also caused a significant increase of calcein-AM uptake in germ free cand humanized mice colonoids compared to the control (Fig. 1b and 2b). Overall, it was noted that the uptake of calcein-AM in humanized mice colonoids was higher than that in germ free mice colonoids. We determined the mean fluorescence in the organoids generated due to calcein-AM uptake in the organoids with and without to the treatment with inhibitors (Fig. 1d and 2d).

3.2 EFFECTS OF VARIOUS INHIBITORS ON PHEOPHORBIDE-A ACCUMULATION IN ORGANOIDS

The effect of BCRP inhibitor novobiocin on the accumulation of pheophorbide-A in the colonoids was studied. The intense fluorescence signals of pheophorbide-A when added alone was observed real-time by confocal microscopy 0.5 h after incubation with a slight contrast against those in the outer space of organoids as seen in Fig. 4a and 5a. In presence of novobiocin, we observed an increase in accumulation of pheophorbide-A over control in both germ free colonoids and humanized mice colonoids as seen in Fig. 4b and Fig.5b. The rate of transport was suppressed in the presence of novobiocin at 15 μM and can be seen in the higher fluorescent intensity of the area outside of the lumen, indicating the diffusion of Pheophorbide A. Overall, it was noted that the uptake of pheophorbide A in humanized mice colonoids was higher than that of germ free colonoids. We determined the mean fluorescence intensities of these organoids with and without pheophorbide-A treatments and presented as rFig. 4c and Fig. 5c.

3.3 ANTI-P-GLYCOPROTEIN IMMUNOHISTOCHEMISTRY

We also tested the expression of P-gp in colonic tissue explants. Immunostaining with the antibody, capable of labeling Abcb1a on the apical surface of epithelium in the colonic tissue, revealed predominant expression of the P-gp. There was nearly 8-fold increase in the expression of P-gp in the explants from humanized (Fig. 3b) and conventional mice (Fig. 3c) compared to the germ free mice (Fig. 3a). A bar chart of the P-gp expression was presented in Fig. 3d.

3.4 ANTI-BREAST CANCER RESISTANCE PROTEIN (BCRP) IMMUNOHISTOCHEMISTRY

We also tested the expression of BCRP in the colonic tissue explants. Immunostaining with the antibody, capable of labeling ABCG2 revealed predominant expression of the transporter on the apical surface of epithelium in the colonic tissue,. There was an increased expression of BCRP in humanized (Fig. 6b)and conventional mice (Fig. 6bc) based on the mean florescence intensities (Fig. 6d) as compared to germ free mice (Fig. 6a) in colonic tissue explants.

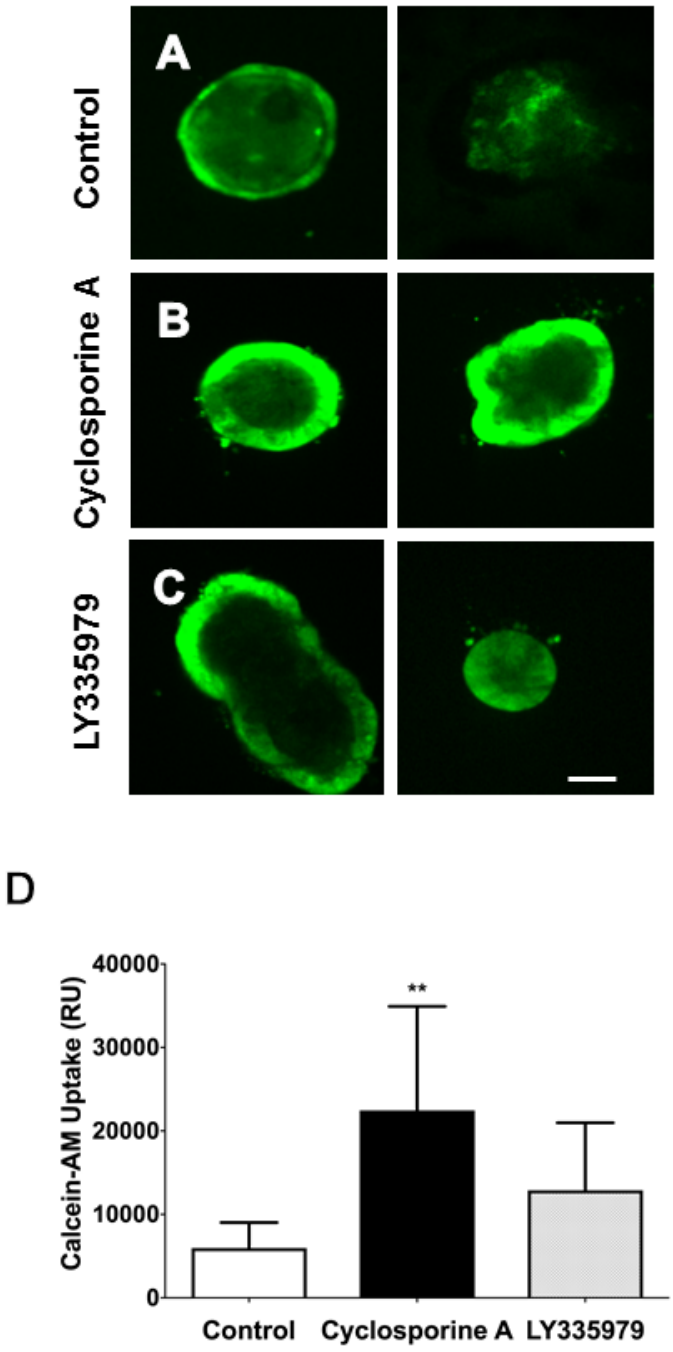

Figure 1. Confocal microscopy images showing accumulation of calcein after 60 minutes incubation at 37°C in Germ free colonoids. Shown are the levels and localization of (A) calcein (0.5 µM); (B) calcein after 60 minutes preincubation for 30 minutes with cyclosporine A (5 µM); (C) preincubation for 30 minutes with LY335979 (1 µM). The higher fluorescence intensity around the rim in presence of inhibitors indicates calcein-AM efflux activity. (D) Effect of various drug efflux protein inhibitors on the accumulation of calcein-AM in the Germ free colonoids. Mean ± SE. One-way ANOVA followed by Bonferroni's multiple comparison test; *P > 0.1, not significantly different from control value; **P < 0.05; ***P < 0.001, significantly different from control value.

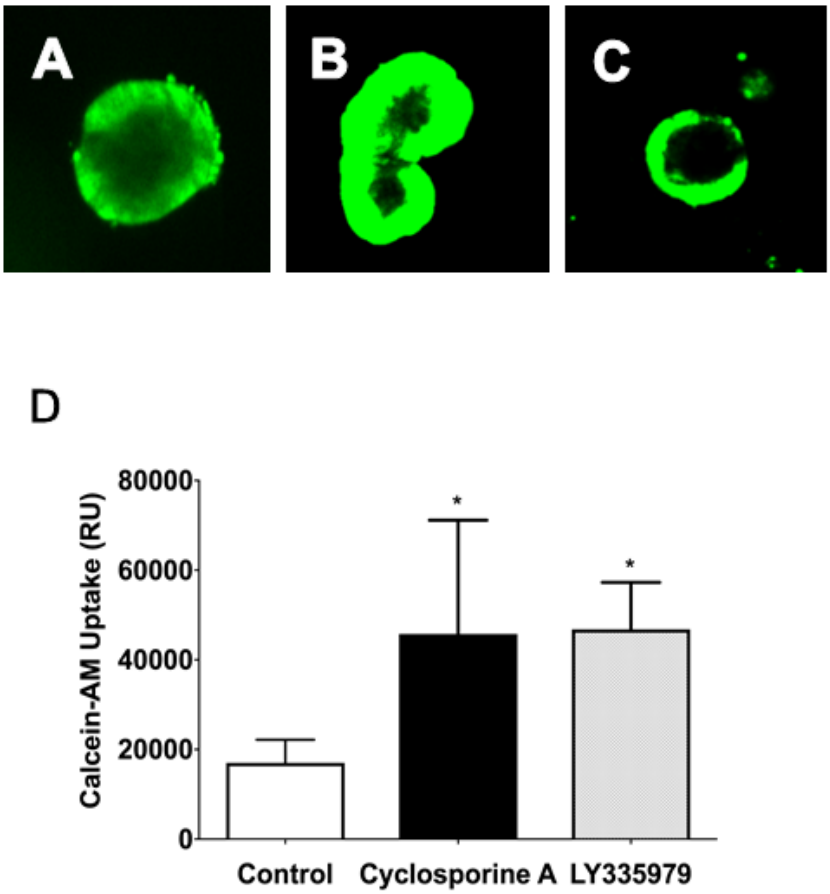

Figure 2. Confocal microscopy images showing accumulation of calcein after 60 minutes incubation at 37°C in Humanized mice colonoids. Shown are the levels and localization of (A) calcein (0.5 μM); (B) calcein after 60 minutes preincubation for 30 minutes with cyclosporine A (5 μM); (C) preincubation for 30 minutes with LY335979 (1 μM). The higher fluorescence intensity around the rim in presence of inhibitors indicates calcein-AM efflux activity. (D) Effect of various drug efflux protein inhibitors on the accumulation of calcein-AM in the Humanized mice colonoids. One-way ANOVA followed by Bonferroni's multiple comparison test; Mean ± SE. *P > 0.1, not significantly different from control value; **P < 0.05; ***P < 0.001, significantly different from control value.

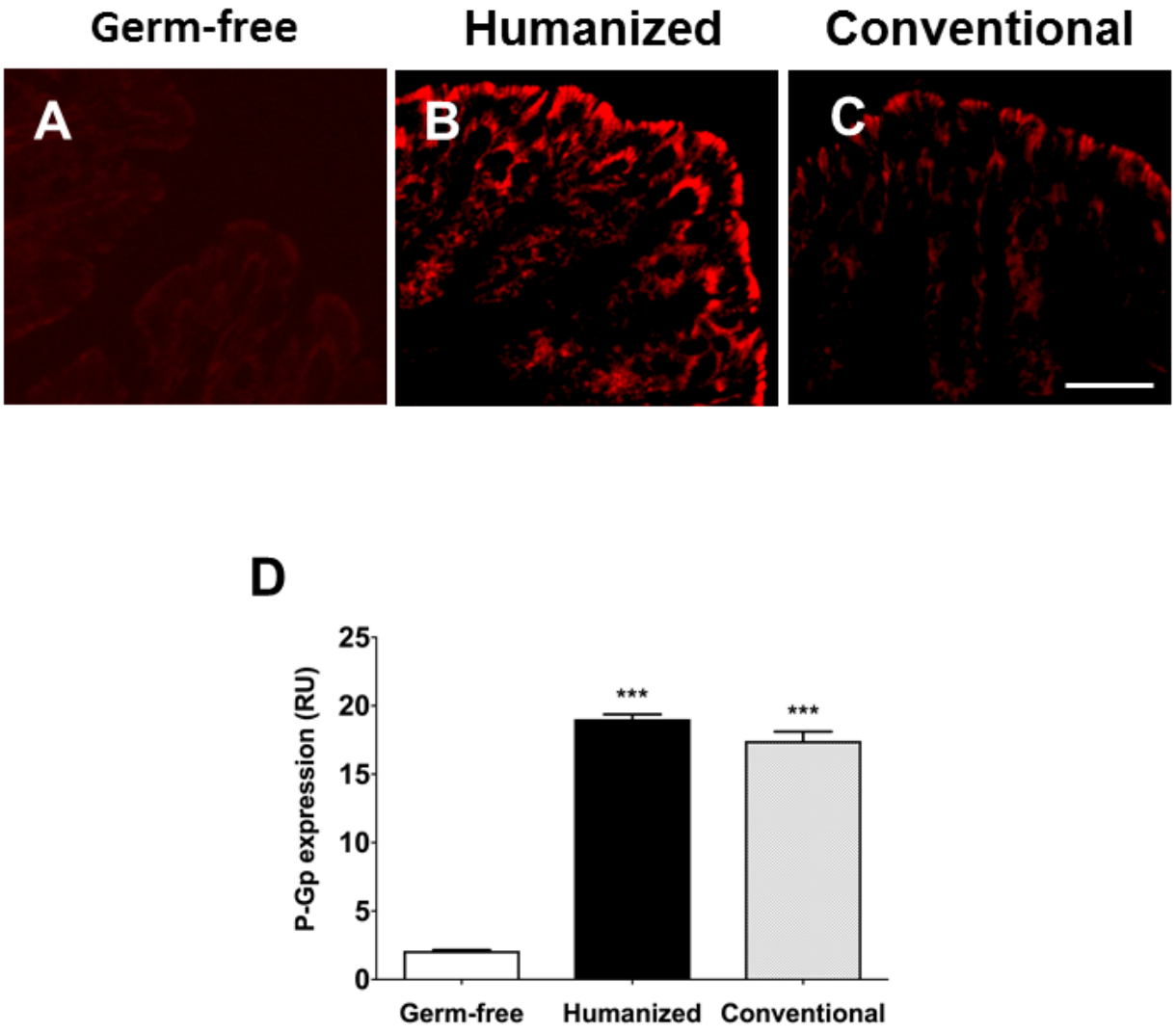

Figure 3. Physiological expression of P-gp proteins by Immunohistochemistry with an anti-Mdr1 antibody can be seen on the edges of the apical side. can be seen in (A) Germ free mice Colon; (B) Humanized mice Colon; (C) Conventional mice Colon. Scale bars, 100 µm. (D) Calculated mean intensity differences of expression of P-glycoprotein in Germ free (GF), Humanized (Hum) and Conventional (Conv) Colonic tissue explants. Mean ± SE. One-way ANOVA followed by Bonferroni's multiple comparison test; *P > 0.1, not significantly different; **P < 0.05; ***P < 0.001, significantly different from Germ free colon value.

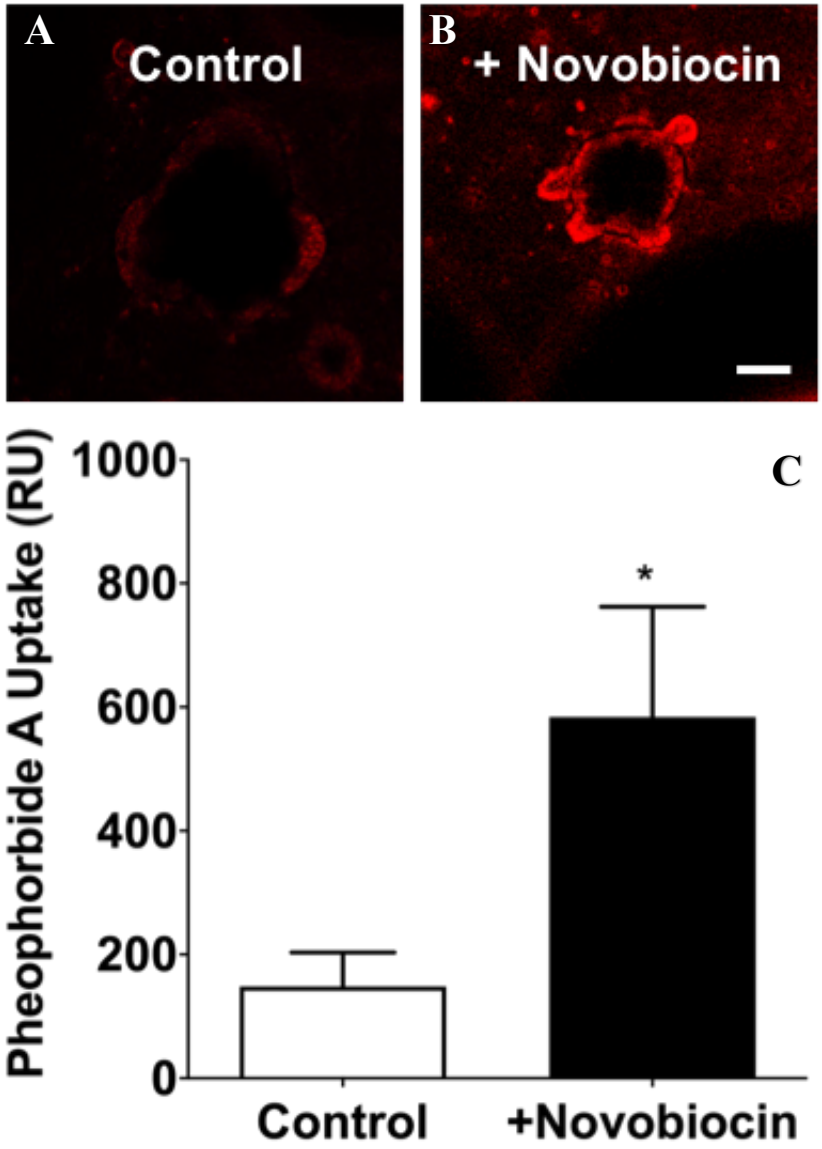

Figure 4. Confocal microscopy images showing accumulation of Pheophorbide A (15 μM) after 30 min incubation at 37°C in germ free mouse colonoids. Extent of pheophorbide A accumulation increased after 30 min preincubation with Novobiocin (15 μM). The data is expressed as mean ± SEM. Student's t-test, *P < 0.05 control versus novobiocin treatment. Scale bar 50 μm.

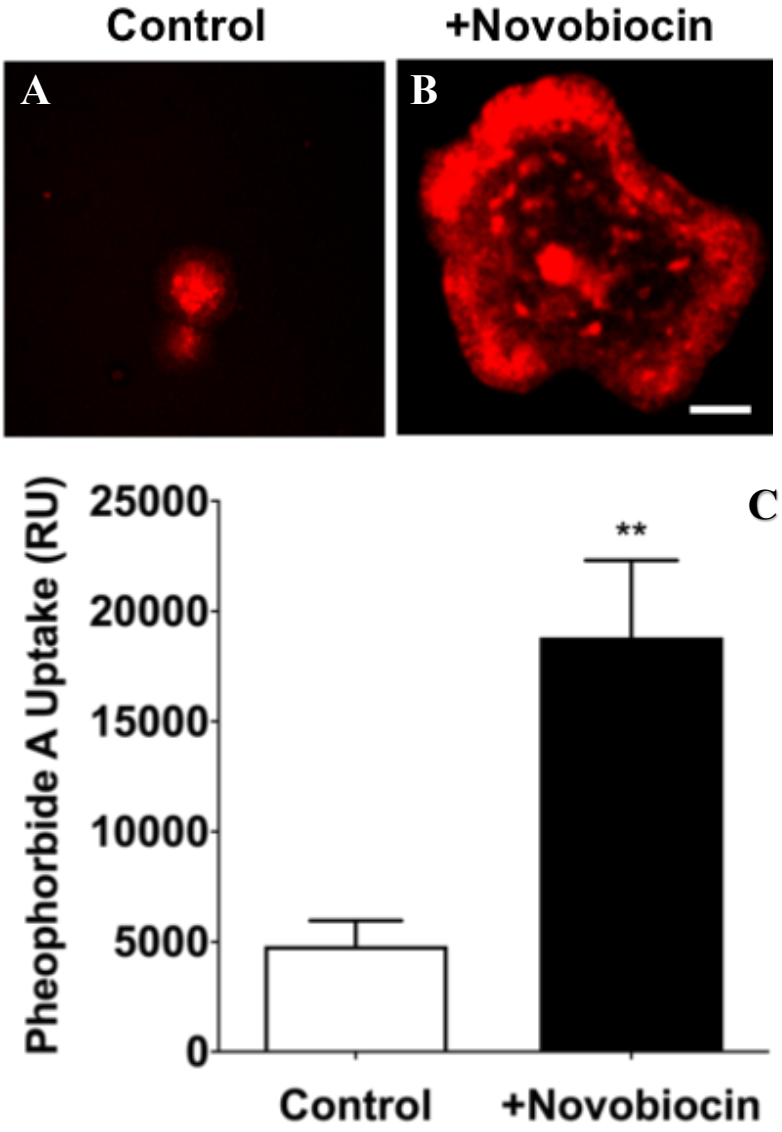

Figure 5. Confocal microscopy images showing accumulation of Pheophorbide A (15 µM) after 30 min incubation at 37°C in humanized mouse colonoids. Extent of pheophorbide A accumulation increased after 30 min preincubation with Novobiocin (15 µM). The data is expressed as mean ± SEM. Student's t-test, *P < 0.05 control versus novobiocin treatment.

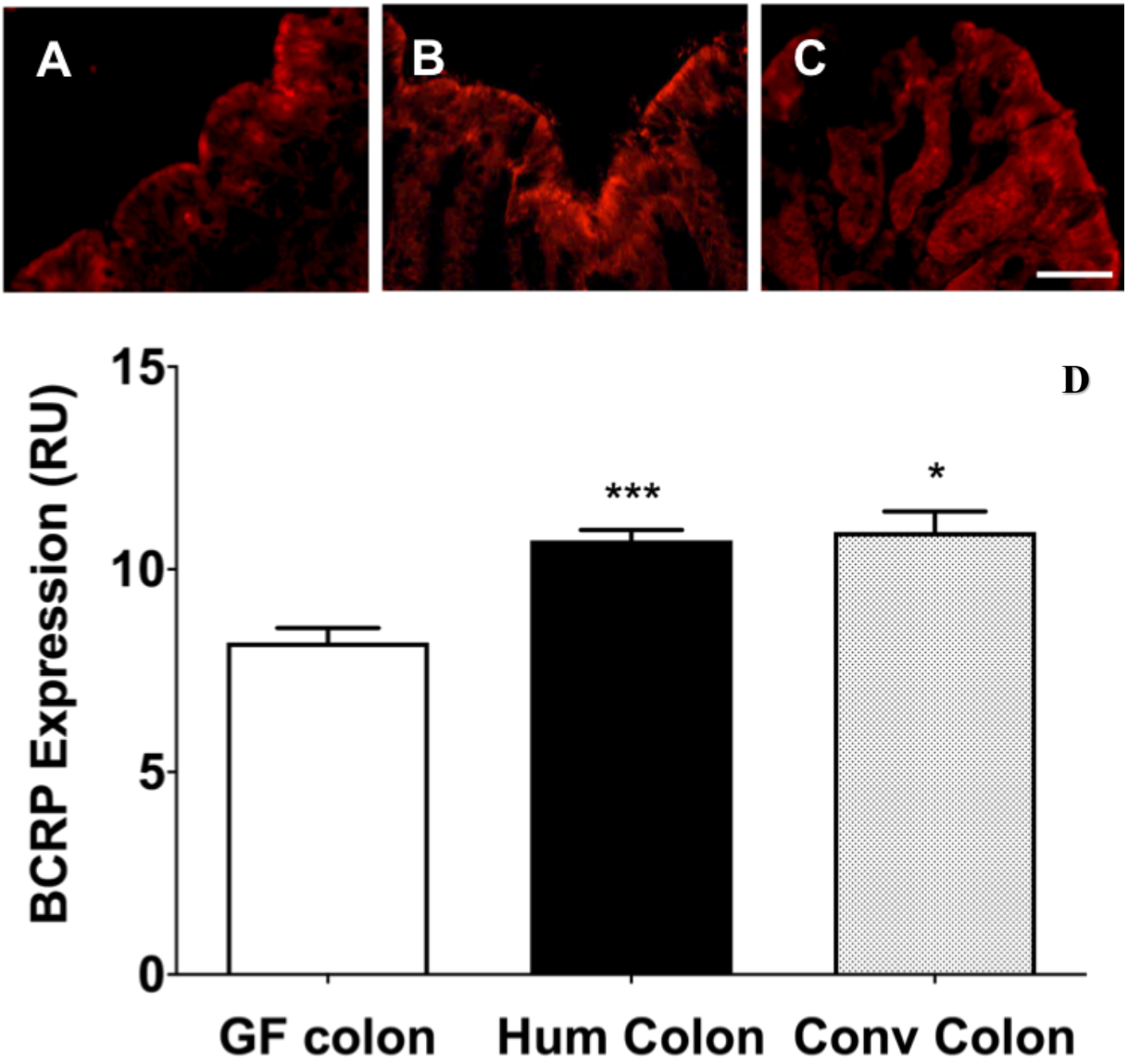

Figure 6. Expression of BCRP proteins by Immunohistochemistry with an anti-BCRP antibody on the apical side of the epithelium. observed in (A) Germ free mice colon; (B) Humanized mice colon; (C) Conventional mice colon. Scale bars, 50 µm. (D) Calculated mean intensity differences of expression of BCRP proteins in Germ free, Humanized and Conventional Colonic tissue explants. The data is expressed as mean ± SEM. One-way ANOVA followed by Bonferroni's multiple comparison test; ***$P < 0.001$ germ free versus humanized mouse colon and *$P < 0.05$ germ free versus conventional mouse colon.

DISCUSSION

In this study, we investigated the effect of gut bacteria on the expression of efflux transporters, P-gp and BCRP, and evaluated the cellular uptake of the fluorescent substrates of these efflux transporters. The role of gut microbiota as a potential regulator of gastrointestinal physiology has been well documented. Previous studies conducted in our lab demonstrated that the colon of germ-free mice is highly permeable to passive diffusion markers such as ^{14}C-PEG4000 than that of the conventional mice. The mucosal barrier integrity was restored when the GI tract of germ-free mice was inoculated with healthy human microbiome (referred to as the humanized mice), thus suggesting the important role of the colonic bacteria in modulating the permeability of gastrointestinal mucosa to small molecules. Based on these observations, we hypothesized that gut microbiota influences the expression and function of efflux transporters, such as P-gp and BCRP in the GI mucosa, which restrict the absorption of xenobiotics, including the absorption of orally administered drugs. Our findings highlight that in the absence of bacteria, i.e. in germ free mice, the expression of P-gp and BCRP was lower in the colon but elevated in the presence of gut bacteria in humanized mice. The relationship between the expression of efflux transporters and their function was confirmed by the efflux activity of the fluorescent substrates of the efflux transporters in the colonoids.

To study these differences, we developed a novel experimental system by culturing three-dimensional mouse colonoids from tissues resected from germ-free, humanized and conventional mice. This system takes advantage of the unique ability of a single intestinal Lgr5-CBC stem cell to grow into structures that illustrates the self-renewing intestinal epithelium that closely resembles the crypt villus architecture of the gut.[29] The organoid

model provides the advantage of maintaining spatial relationship of various cell types and for the most part recapitulates the transporter expression observed in vivo. The use of excised tissues to study the permeability in small intestine and colon with or without its underlying layers in-vitro has been attempted in the past but a major disadvantage of this method is the limited viability of tissues when isolated. Additionally, several studies have shown the correlation between in vitro permeability values in Caco-2 assays and parameters of in-vivo absorption. However, discrepancies due to the variability in results is seen in such assays. A major reason for this is cellular heterogeneity due to the phenotypic drift seen under different conditions.[2] The organoid model addresses some of the disadvantages of using tissue explants or Caco-2 cell monolayers to study drug permeability due to their inconsistencies observed in different culture conditions[4]. Additionally, Caco-2 like cell systems require a bi-directional permeability assessment for efflux transport calculations to study the difference in concentrations across a membrane which is a tedious process.[2,4]

In this regard, the uniqueness of our system is the use of purified, non-transformed intestinal epithelial cells keeping their physiological expression and localization of P-gp. The ability of organoids to provide insightful results owing to their geometry and physiological characteristics like the presence of tight junctions and packed inner spaces that closely resembles in-vivo characteristics makes organoids useful. For example, the study of baso-apical transport is a unidirectional exercise as organoids are tightly packed and the difference in inward transport and outward diffusion provides intuitive information or studying the expression of proteins through immunofluorescence.[2] The availability of such models for transport assays would build a basis for a variety of applications including

the screening of inducers/inhibitors of P-gp in normal intestinal cells, or assessing the P-gp function in individual human specimens and possibly provide a new, more efficient means of using such assays for research.

In this work, we studied the expression of efflux transporters and their correlation with the transport of substrates using germ-free and humanized mouse colonoids. Typically, we assume that the substrate concentration in the tightly-closed lumen of the organoid is determined by two mechanisms at the apical cell membrane interface; active inward transport (basal-to-apical direction) and passive bidirectional diffusion.[2] The presence of efflux transporters increases the active transport in the basal to apical direction and subsequently reduces the uptake of substrates from the lumen. Our data shows that the Calcein-AM uptake was lower for germ-free mice colonoids than those obtained from humanized mice in control samples. These results were further verified by data obtained from the immunohistochemistry studies that showed a significantly higher expression of P-gp in humanized and conventional mouse mucosal explants as compared to those from germ-free mice. These results demonstrated the association between the efflux transporter expression and its impact on the cellular uptake and transport. We hypothesize that the higher P-gp expression in humanized mice generates a higher basal to apical efflux resulting in higher mean intensities within the lumen. Similarly, lower expression of P-gp results in lower efflux activity. The degree of inhibition of P-gp activity was quantitated by measuring the intracellular fluorescence. Inhibition of P-gp, allows for greater cellular transport of molecules and thus we see lower fluorescence within the lumen due to reduced active efflux in the basal to apical direction. The major differentiating factor in these mouse

models is the bacterial content in the gut, and thus these functional and expressional differences can be attributed to the presence of gut bacteria.

Similar to P-gp, we hypothesized association between the expression and efflux mediated by BCRP. We hypothesize that the higher BCRP expression in humanized mice creates a higher basal to apical efflux resulting in higher mean intensities within the lumen. Similarly, lower expression of BCRP results in lower efflux activity. The degree of inhibition of BCRP activity was quantified by measuring the intracellular fluorescence. Inhibition of BCRP, resulted in lower fluorescence within the lumen due to reduced efflux of in the basal to apical direction.

The major differentiating factor in these mouse models is the bacterial content in the gut, and thus these functional and expressional differences can be attributed to the presence of gut bacteria. Bacteria or bacterial products are known to play a major role in altering physiological functions within the body like altering tight junctions and changing transepithelial electrical resistance across the cell monolayers.[40] However, there are certain limitations of using these ex-vivo models to study the aforementioned effects of bacteria and thus cannot completely replace in-vivo studies or clinical studies. Germ-free mice are very good models to study the mechanistic differences and understand the physiological effects of bacterial strains but the absence of bacteria results in an immature immune system in these mice and thus the results cannot be directly translated to humans as several other factors need to be studied. In humanized mice on the other hand, bacteria are colonized for a short duration of time in mice that have been in isolation for a long period of time thus creating a cause for bias. Despite these limitations, our studies were conducted in parallel in these mice and thus forms a strong basis for one on one comparisons.

Additionally, several studies have shown the benefits of using these models for preclinical research and thus establishing them as effective models for mechanistic investigations.[9,40]

We have shown that in germ free mice, the permeability of a passive diffusion marker such as (^{14}C-PEG4000) was higher than in conventional or humanized mice. These results suggest that bacteria play a very important role in altering gut permeability by modulating the expression of tight junctional proteins and efflux transporters. In addition, taking advantage of the real-time imaging, we described a method to evaluate the dynamics of P-gp and BCRP-mediated efflux in gut organoids in vitro. This system could be helpful in investigating the physiological function and screening of inhibitors/inducers of efflux transporters and may serve as a novel tool to investigate the bioavailability of orally administered drugs.

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