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PRINCIPAL INVESTIGATOR: Matthew Grisham, PhD.

CONTRACTING ORGANIZATION: Texas Tech University Health Sciences Center, Lubbock, TX.  
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| 6. AUTHOR(S)<br><br>Matthew B. Grisham, PhD.<br><br>E-Mail: <a href="mailto:matthew.grisham@ttuhsc.edu">matthew.grisham@ttuhsc.edu</a>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |              |                         |                            | 5d. PROJECT NUMBER                       |                                           |
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| 14. ABSTRACT: The overall objective of this proposal is to evaluate the therapeutic efficacy of human, bone marrow-derived mesenchymal stem cells (hMSCs) in a mouse model of inflammatory bowel disease (IBD). As documented in our previous Progress Reports, we encountered a number of major problems following relocation to our current institution that significantly delayed the initiation of the project. By the far the most perplexing and time-consuming problem was the loss of disease phenotype of our mouse model of IBD that we had used for several years during my tenure at LSU Health Sciences Center (LSUHSC). We hypothesized that the loss of phenotype was due to significant differences in the microbial composition of mice housed at the two animal facilities. Thus began, a 3 year process to "re-derive" our original mouse model of chronic colitis. To do this, we undertook a systematic study to quantify and compare the intestinal microbiota within "disease susceptible" mice housed at LSUSHC vs. "disease-resistant" mice that were housed at Texas Tech University HSC. These studies generated new and exciting data that have greatly impacted our understanding of host-microbe interactions in experimental IBD. Using these new data, we were able reestablish the disease phenotype with mice developing robust colitis. In addition, we have utilized these new data to evaluate the therapeutic efficacy of hMSCs using a more <i>clinically-relevant</i> treatment modality in our new mouse model of IBD. Surprisingly, we found that intravenous administration of hMSC, at doses used in clinical studies to treat humans with IBD, failed to attenuate the development of chronic colitis. We conclude that the rationale for the use of MSCs to treat patients with IBD may need to be re-examined. |              |                         |                            |                                          |                                           |
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## 1. INTRODUCTION

The inflammatory bowel diseases (IBD; Crohn's disease; ulcerative colitis) are chronic inflammatory disorders of the small bowel and/or colon that affects approximately 1.5 million people in the US with a calculated *annual cost* for both medical expenses and work loss of almost \$4 billion dollars. A recent study analyzing the Department of Veterans Affairs database from 1975-2006 reports that although rates of hospitalization for ulcerative colitis (UC) and Crohn's disease (CD) have begun to stabilize over the past few years, there has been a disproportionate increase in rates of hospitalizations for nonwhite vs. white US military veterans for both UC and CD. Currently, there are only a handful of medical treatments available to treat these debilitating inflammatory disorders with only a few new therapies projected to be available in the near future. Thus, there is a clear need for the development of additional therapeutic agents to treat patients with IBD. A great deal of excitement has been generated from recent studies demonstrating that adoptive transfer of syngeneic, allogeneic or xenogeneic (*human*) MSCs suppress the inflammation and tissue injury observed in animal models of autoimmune encephalomyelitis, allograft rejection, arthritis and graft vs. host disease. Because MSCs can be grown and expanded *in vitro* and exert their immuno-regulatory activity across major histocompatibility complex barriers *in vivo*, we are in the unique position to evaluate the therapeutic efficacy of *human* MSCs in our mouse model IBD. The **overall objective** of this proposal is to evaluate the therapeutic efficacy of *ex vivo*-generated, bone marrow-derived human MSCs in a well-characterized mouse model of *chronic* colonic inflammation. *Hypothesis*: We propose that *ex vivo*-generated MSCs suppress chronic gut inflammation by homing to the mesenteric lymph nodes (MLNs) and/or colonic lamina propria where MSC-derived TGF $\beta$  suppresses/limits the generation of colitogenic T-cells and/or induces the formation of IL-10-producing Tregs. In order to test this hypothesis we propose the following three specific aims: a) Evaluate the ability of human MSCs to suppress the induction of chronic gut inflammation; b) Determine the therapeutic efficacy of human MSCs in reversing preexisting colitis; and c) Define the immuno-regulatory mechanisms utilized by MSCs to attenuate chronic colitis.

## 2. KEYWORDS

inflammatory bowel disease; microbiota; antibiotics; mesenchymal stem cells; Tregs; IL-10, TGF $\beta$ ; colitis; intestinal inflammation; immuno-suppression.

## 3. ACCOMPLISHMENTS

### *Major Goals of the Project*

**Task 1.** Evaluate the ability of human MSCs to suppress the induction of chronic gut inflammation.

**Task 2.** Determine the therapeutic efficacy of human MSCs in attenuating preexisting colitis.

**Task 3.** Define the immuno-regulatory mechanisms utilized by MSCs to attenuate chronic.

As documented in our three previous Progress Reports, we encountered a number of major problems following our relocation to Texas Tech University Health Sciences Center that significantly delayed the initiation of the project. By the far the most perplexing and time-

consuming problem was the loss of disease phenotype of our mouse model of IBD that we had used successfully for more than 16 years during my tenure at LSU Health Sciences Center. We hypothesized that the loss of phenotype was due to significant differences in the microbial composition of mice housed at the two animal facilities. Indeed, this situation has been described by other investigators using different mouse models of chronic disease who have changed institutions and is thought to be a major reason for irreproducible results of animal models at different institutions. Thus began a long and arduous process to attempt to “re-derive” our original mouse model of chronic colitis. To do this, we began a systematic study to quantify and compare the intestinal microbiota that reside within “*disease susceptible*” RAG<sup>-/-</sup> mice housed at LSUSHC vs. “*disease-resistant*” RAG<sup>-/-</sup> that were housed at TTUHSC. It was our hope that by understanding the differences in intestinal bacterial populations in these two groups of mice, we might be able to manipulate/convert the resistant mice at TTUHSC to a more susceptible phenotype similar to the mice housed at LSUHSC. If successful, we would be able to complete some of the original objectives outlined in the proposal. Unfortunately, the microbial and bioinformatics studies as well as the subsequent model re-derivation required more than three years to complete. Nevertheless, these studies generated new and exciting findings that have greatly impacted our understanding of host-microbe interactions in experimental IBD. In addition, we have utilized these new information to evaluate the therapeutic efficacy of human MSCs using a more *clinically-relevant* treatment modality in our new mouse model of IBD. Described below is a summary of our *major accomplishments* during the funding period. Unless otherwise stated, all methods, analyses and figures described in the body of this Progress Report can be found in the attached manuscript that has recently been accepted for publication in Inflammatory Bowel Diseases ([Reinoso Webb et. al., 2017, Inflammatory Bowel Diseases, In press \(Appendix Item 1\)](#)).

### ***Specific Microbiota are Differentially Overrepresented in Susceptible vs. Resistant Mice***

The loss of phenotype in our mouse model coupled to the lack of any detailed microbiological analyses in the T cell transfer model of chronic colitis prompted us to quantify and compare the colonic microbiota in healthy and colitic mice obtained from TTUHSC and LSUHSC and determine whether manipulation of colonic microbiota may alter the incidence and/or severity of chronic colitis at our current institution (TTUHSC). To our knowledge, this is ***the first*** systematic, bioinformatics analyses performed on the widely-used T cell transfer model of IBD. Data generated from these studies revealed specific and highly significant differences between the microbial compositions associated with susceptible mice housed at LSUHSC vs. the resistant animals housed at TTUHSC. Indeed, Principal Coordinate Analysis with weighted Unifrac of mouse fecal microbiota revealed a clear separation/difference between the two groups indicating significant differences between the operational taxonomic unit (OTU) composition and relative abundance of the microbiota ([Figure 2A; Appendix Item 1](#)). In addition, DESeq2 analysis identified large and significant differences (< 0.01 multiple comparison adjusted p-value) *within and between* groups at the genus level, including differences in strain and/or species that may otherwise be missed when looking at higher taxonomic classifications. The vast majority of differentially expressed OTUs in feces from LSUHSC mice were associated with Firmicutes with fewer genera assigned to Bacteroidetes, Actinobacteria and Teneicutes ([Figure 2B; Appendix Item 1](#)). The most overrepresented genera that were differentially over represented in LSUHSC vs. TTUHSC mice (>10 log<sub>2</sub> fold change) were *Ruminococcus*, *Bifidobacterium*, *Clostridium sensu stricto*, *Alistipes*, *Anaeroplasma* and *Barnesiella* ([Figure 2B; Appendix Item 1](#)). (Note: a

10 log<sub>2</sub> fold change represents an increase of 2<sup>10</sup> or a 1,024 fold increase in OTU expression). Although it is not apparent which of the 6 overrepresented genera in LSUHSC mice converts susceptibility to induction of colitis, there are reports demonstrating that certain mucolytic bacteria from the genus *Ruminococcus* (e.g. *R. gnavus* and *R. torques*) are increased in the mucosa of patients with IBD. We are currently attempting to identify the specific species within this and other genera that are overrepresented in the highly susceptible LSUHSC animals.

In addition to the unique genera that colonize healthy RAG<sup>-/-</sup> mice housed at LSUHSC, we observed 4 genera that were differentially overrepresented and highly associated with microbiota obtained from resistant TTUHSC mice including *Blautia*, *Extibacter*, *Dorea*, *Flavonifractor* and *Intestimonas* (Figure 2B; Appendix Item 1). Very little is known about the relationship between these genera and susceptibility to gut inflammation; however, *Blautia* has been shown to possess potent *anti-inflammatory activity in vivo*. In fact, this specific genus has been shown to be associated with *reduced risk* of human graft-versus-host-disease, as well as *improved outcomes* in colorectal cancer, inflammatory pouchitis and liver cirrhosis. We speculate that future studies may reveal immunosuppressive/protective properties of specific species within these genera that may be useful in suppressing intestinal inflammation.

### ***Robust Disease Develops in RAG<sup>-/-</sup> Mice Housed at TTUHSC Following Exposure to Microbiota from RAG<sup>-/-</sup> Animals Housed at LSUHSC***

Another novel observation we made during these studies was that robust disease will in fact develop in *resistant* RAG<sup>-/-</sup> mice housed at TTUHSC, provided that T cell transfer occurs and animals are housed at the LSUHSC animal facility for an initial 2 week period prior to their transport to TTUHSC (Figure S1; Appendix Item 1). These studies suggested that once the initial immunological interactions occur between naïve CD4<sup>+</sup> T cells and “appropriate” microbial antigens, induction and progression of chronic disease continues unabated over the ensuing 6 week observation period *irrespective of the animal care facility*. How this occurs is only a matter of speculation; however, we suggest a scenario where the initial immune responses that occur between engrafted T cells and microbial antigens within the first 2 weeks, alters dramatically the composition of the commensal microbiota. This dysbiosis would continue to develop unabated over the ensuing weeks resulting in the expansion of pathobionts that are ultimately responsible for the induction of chronic colitis. Indeed, it is quite possible that inflammation-induced dysbiosis would both initiate disease as well as perpetuate chronic colitis irrespective of the housing environment. If these hypotheses are correct, then one would predict that colonization of healthy RAG<sup>-/-</sup> mice at TTUHSC with feces obtained from healthy RAG<sup>-/-</sup> mice housed at LSUHSC would render these mice *more susceptible* to induction of chronic colitis. In fact, that is exactly what we observed. We found that transfer of T cells into RAG<sup>-/-</sup> housed at TTUHSC that were first colonized with feces from healthy or colitic RAG<sup>-/-</sup> housed at LSUHSC induced robust colitis (Figures 6 and 7; Appendix Item 1). These data appear to support the following conclusions: a) the bacterial composition of the commensal microbiota determines whether or not robust disease will develop following T cell transfer and b) a relatively brief exposure (2 weeks) of T cell engrafted mice to “disease-producing”, microbial antigens is sufficient to drive the development of chronic colitis that is independent of the housing conditions.

### ***Chronic Gut Inflammation is Highly Associated with Dramatic and Significant Increases in Specific Genera within the Phyla Bacteroidetes and Verrucomicrobia but not Proteobacteria***

One of the most consistent observations reported in patients with IBD and in some mouse models of IBD is that intestinal inflammation is highly associated with the expansion of Proteobacteria with a concomitant reduction of Firmicutes. Surprisingly, we failed to observe an expansion of Proteobacteria and its associated families/species (e.g. *Enterobacteriaceae*, *Helicobacteraceae*, *E. coli* etc) in mice with chronic colitis (Figures 3 and 4; Appendix Item 1). On the contrary, we observed that the 4 of the 7 most overrepresented genera in colitic mice housed at LSUHSC were members of the phyla Firmicutes ( $>10$  log<sub>2</sub> fold change; Figures 3B and 4B; Appendix Item 1). However, the *largest increases* in genera (i.e. OTU expression) in these colitic mice were found to be members of the phyla Bacteroidetes (e.g. Muribaculum and Bacteroides) and Verrucomicrobia (e.g. Akkermansia). Muribaculum OTU expression was found to be increased 1,000-32,000 fold vs. healthy mice; Figure 3B; Appendix Item 1). Indeed, a particularly novel finding was the presence of at least 9 different species and/or strains of Muribaculum in the feces from colitic mice (Figure 3B; Appendix Item 1). In addition, we also observed  $>1,000$  fold increase in expression of Bacteroides OTUs vs healthy mice. It is known that some members of Bacteroides are capable of digesting mucin and inducing chronic colitis in antibiotic-treated, genetically-susceptible mice. In fact, we report, *for the first time* a dramatic and significant increase in OTU expression of the Verrucomicrobia member Akkermansia ( $>32,000$  fold increase compared to healthy mice; Figure 3B; Appendix Item 1). The only known species of this genera is the mucin-degrading bacterium Akkermansia muciniphila. This bacterium has been shown to be increased in experimental and human IBD. Although it is not clear whether the expansion of these bacteria alone or in combination with other overrepresented bacteria (i.e. Muribaculum, Bacteroides) are responsible for the induction and/or perpetuation of chronic disease, it may be possible to test the role of these bacteria in future studies using gnotobiotic approaches.

### ***Antibiotic Administration but not Fecal Microbial Transplantation Attenuates Preexisting Colitis***

It is well-known that commensal bacteria are required for induction of chronic intestinal inflammation in most of the mouse models of IBD. To our knowledge, *no studies* have been performed evaluating the *therapeutic efficacy* of broad spectrum antibiotic (ABX) treatment in a more clinically-relevant situation where mice present with preexisting colitis. Data obtained in this study demonstrate, *for the first time*, that administration of an ABX cocktail to mice with *preexisting* disease remarkably attenuates colonic inflammation (Figure 9; Appendix Item 1). In addition, while ABX-treated mice appeared to have many fewer bacteria than controls, we observed a significant and differential expansion of Proteobacteria, as well as loss of Bacteroidetes, when compared to untreated controls (Figure 9; Appendix Item 1). At first glance, these data appear to be counter intuitive as many human and animal studies suggest that expansion of certain genera within Proteobacteria are strongly associated with disease. Although the reasons for this apparent paradox are not known, it may be that one or more of the overrepresented genera following ABX [e.g. *Lactobacillus* (phyla Firmicutes); Table 1; Appendix Item 1] counteract the potential proinflammatory activity of the remaining Proteobacteria. Alternatively, the surviving members of Proteobacteria may not be disease-producing pathobionts or they simply cannot induce chronic colitis at their reduced numbers following ABX treatment. Nevertheless, ABX treatment may be effective in attenuating distal bowel disease.

Another objective of the current study was to assess the therapeutic efficacy of fecal microbial transplant (FMT) in mice with *preexisting* disease. Data from these studies demonstrate, **for the first time**, that FMT administration alone did not attenuate colonic inflammation even when administered multiple times following the onset of disease (Figure 9; Appendix Item 1). Although we observed that the combination of ABX+FMT significantly reduced colonic inflammation, the protective effects were similar to ABX treatment alone suggesting that broad-spectrum antibiotics are much more effective than FMT in treating established disease (Figure 9; Appendix Item 1). We propose that the use of FMT alone may not provide clinically efficacious and/or durable treatment for human IBD.

### ***MSC Administration Fail to Suppress Chronic Gut Inflammation When Administered in Clinically-Relevant Doses***

With the ability to induce more consistent colonic inflammation via transfer of colitic microbiota to RAG<sup>-/-</sup> mice housed at TTUSHC, we began a systematic evaluation of the therapeutic efficacy of human MSCs in this new model. Preliminary data generated at LSUHSC prior to the grant submission demonstrated “*proof of concept*” that weekly injections (**i.p.**) of 5 million human MSCs for six weeks following T cell transfer significantly suppressed the development of chronic colitis in our mouse model of IBD. However, recent clinical studies using human MSCs to treat Crohn’s Disease suggest that the “dose” of MSCs we used for our preliminary studies (equivalent to 166 million/kg for a 30 gram mouse) is far in excess of what would ever be used clinically to treat human IBD. Current clinical studies in humans use different doses of MSCs that are administered in 2 infusions (**i.v.**) per week for 2 consecutive weeks (a total of 4 injections). One of the more recent clinical studies use either a “low dose” (2 million/kg per injection) or “high dose” (4 million/kg per injection) MSCs to treat Crohn’s disease (<http://clinicaltrials.gov/show/NCT00294112>). Therefore, we followed the human clinical trial protocol in which the mouse equivalent of “high dose” MSCs (4 million/kg per injection) were used. Using known body surface area differences between human and mouse, the following formula was used to convert human equivalent dose (HED) to animal dose:

*HED = [Animal dose] [Animal km/Human km]; Where Animal km and Human km are known (please refer to Reagan-Shaw et. al. FASEB J. 22:659, 2007)*

Using this formula, we calculated that the mouse equivalent of “high dose” MSCs (4 million/kg per injection) is 1.3 million per injection. Chronic colitis was induced via adoptive transfer of 5x10<sup>5</sup> CD45RB<sup>high</sup> T cells into RAG<sup>-/-</sup> mice that had been colonized with colitic feces one week prior to T cell transfer. Human MSCs (~1.3 million cells in 0.5 mls PBS) or PBS (0.5 mls) was injected (**i.v.**) 2 times per week for 2 consecutive weeks (a total of 4 injections) beginning 2 weeks post T cell transfer (N=12 for PBS and N=9 for hMSCs). At 7 weeks post T-cell transfer, mice were euthanized and colons removed for blinded histopathological and flow cytometric analyses. We found no significant differences in body weight between the 2 groups over the 7 week observation period (Appendix Item 2; Figure 1). Macroscopic evidence of colonic inflammation was assessed using our established clinical scoring criteria based on the following: normal appearing colons were assigned a score of 0, mild bowel wall thickening in the absence of visible hyperemia was assigned a score of 1, moderate bowel wall thickening and hyperemia was given a score of 2, severe bowel wall thickening with rigidity and marked hyperemia was assigned

a score of 3 and severe bowel wall thickening with rigidity, hyperemia and colonic adhesions was given a score of 4. We observed moderate colonic inflammation in the colons from both groups of mice that were not significantly different ([Appendix Item 2; Figure 2](#)). These results were similar to what we observed for colonic weight-to-length ratios which provides a quantitative index of inflammation ([Appendix Item 2; Figure 2](#)). Furthermore, treatment of mice with a *clinically-relevant dose* of MSCs did *not* significantly attenuate colonic inflammation as assessed by blinded histopathological analysis ([Appendix Item 3; Figure 3](#)). Finally, treatment of mice with MSCs had no significant effect on the numbers of colonic T cells, PMNs and macrophages ([Appendix Item 2; Figure 4](#)). At first glance, these data are a bit surprising given the number of animal studies reporting protective effects of these immunomodulatory cells in experimental IBD. It should be noted however, that virtually all of these studies have used large numbers of MSCs in erosive, self-limiting (i.e. acute) models of **chemically-induced** colitis. Based upon our studies, we believe the the rationale for the use of MSCs to treat patients with chronic IBD may need to be reexamined. Additional experiments are currently underway to assess the therapeutic efficacy of MSCs when administered following the onset of chronic colitis.

#### Opportunities for Training and Professional Development

Although the primary purpose of this application was not to provide training and professional development, my graduate students and postdoctoral fellows have received extensive training in immunology, use of mouse models of IBD and flow cytometry.

#### Dissemination of Results

Data generated from these studies have been presented at the National Crohn's and Colitis meeting held in December, 2014 in Hollywood, FL., the National MHSRS meeting held in August, 2015 in Ft. Lauderdale, FL., the American Association of Immunology held in May, 2016 in Seattle WA and the FASEB Summer Research Meeting held in August, 2017 in Steamboat Springs, CO. In addition, data generated during the funding period were included in a our manuscript that was recently accepted for publication in *Inflammatory Bowel Diseases* (Reinoso Webb et. al., 2017, *Inflammatory Bowel Diseases*, In press-Appendix Item 1).

#### Plans for the Next Funding Period: this is the Final Progress Report

## **4. Impact**

#### Impact on discipline

Data generated from our studies will impact greatly investigators using mouse models of IBD to evaluate therapeutic efficacy of different biologics and cell-based therapies. Our studies provide, for the first time, a systematic study of how the intestinal microbiome affects the onset and severity of chronic colitis in the widely used T cell transfer model of IBD. Our results are particularly important in view of highly publicized *inability* of investigators to **reproduce** published studies demonstrating therapeutic efficacy of novel, small molecules or biologics in mouse models of chronic disease. Furthermore, our data should impact greatly investigators evaluating the therapeutic efficacy of MSCs in mouse models of IBD. Although several preclinical studies have reported protective effects of these immunomodulatory cells in rodent models of IBD, virtually all of these studies have used clinically-questionable doses of MSCs in

erosive, self-limiting (i.e. acute) models of ***chemically-induced*** colitis rather than models of chronic disease. Based upon data obtained from our studies, we believe that the rationale for the use of MSCs to treat patients with chronic IBD may need to be reexamined.

#### Impact on other disciplines

Our results are particularly important to other disciplines that utilize animal models of disease. The inability of investigators within the academic and pharmaceutical/biotechnology communities to reproduce published studies demonstrating therapeutic efficacy of novel, small molecules or biologics in mouse models of disease has received a great deal of discussion in recent months. This is a particularly troubling situation that has garnered a great deal of attention by funding agencies and the publishing community.

#### Impact on technology transfer

Nothing to report

#### Impact on society

Nothing to report

### **5. Changes/Problems**

#### Changes in Approach

No major changes. Based upon new data from clinical studies using human MSCs in the treatment of different inflammatory diseases, we will concentrated our efforts on evaluating the immunosuppressive properties of *clinically-relevant* doses of human MSCs.

#### Problems and Delays

Because of the time required to re-derive and characterized our new mouse model of IBD, we were not able to complete some of the *in vivo* studies outlined in the original proposal.

#### Changes that had a significant impact on expenditures

No major impact on expenditures

#### Changes in use of vertebrate animals

We have reestablished our mouse model of IBD.

### **6. Products**

#### Publications

Reinoso-Webb, C., den Bakker, H., Koboziev, I., Jones-Hall, Y., Kottapali, KR, Ostanin, D.V., Furr, K., Mu, Q., Luo, X. and Grisham, M.B., 2017. Differential Susceptibility to T Cell-Induced Colitis in Mice: Role of the Intestinal Microbiota. *Inflammatory Bowel Diseases (In Press)*.  
Funded by DOD and NIH.

Reinoso-Webb, C., Koboziev, I., Furr, K.L., and Grisham, M.B. 2016. Protective and pro-inflammatory roles of intestinal bacteria. *Pathophysiology*. **23**:67-80. Funded by DOD and NIH.

Fang, K., Grisham, M.B., and Kevil, C.G. 2015. Application of Comparative Transcriptional Genomics to Identify Molecular Targets for Pediatric IBD. *Front Immunol*. **6**:165; Funded by DOD and NIH.

Koboziev, I., Jones-Hall, Y., Valentine, J.F., Webb, C.R., Furr, K.L., and Grisham, M.B. 2015. Use of Humanized Mice to Study the Pathogenesis of Autoimmune and Inflammatory Diseases. *Inflamm. Bowel Dis*. **21**:1652-1673. Funded by DOD and NIH.

### Presentations

Data generated from these studies have been presented at the National Crohn's and Colitis meeting held in December, 2014 in Hollywood, FL., the National MHSRS meeting held in August, 2015 in Ft. Lauderdale, FL.; the Ramsey Distinguished Lecture; Iowa State University; College of Veterinary Medicine in October, 2016, The University of Arkansas for the Medical Sciences; Department of Medicine and Microbiology and Immunology in October, 2016; the American Association of Immunology held in May, 2016 in Seattle WA and the FASEB Summer Research Meeting held in July, 2017 in Steamboat Springs, CO. In addition, data generated during the funding period were included in our manuscript that was recently accepted for publication in *Inflammatory Bowel Diseases* (Reinoso Webb et. al., 2017, *Inflammatory Bowel Diseases*, In press-Appendix Item 1).

## **7. Participants and Other Collaborators**

### Individuals working on project

Name: Matthew Grisham, PhD

Project Role: PI

Nearest person months: 4 months

Contribution: Dr. Grisham is involved in designing, implementing and interpreting the experiments

Funding support: DOD and TTUHSC/State Account

Name: Iurii Koboziev, PhD

Project Role: Postdoctoral fellow

Nearest person months: 6 months

Contribution: Dr. Koboziev performs all cell preparations, molecular and *in vitro* assays and assists with the flow cytometry studies and animal model

Funding Support: DOD and TTUHSC/State Account

Name: Kathryn Furr, MS

Project Role: Laboratory Scientist

Nearest person months: 4 months

Contribution: Ms. Furr is my Lab Manager and Flow Cytometry Core Manager. She performs all flow cytometry experiments; She is generates the mouse model of IBD and assists with some of the MSC cell culture experiments.

Funding Support: DOD and TTUHSC/State Account

Name: Cynthia Reinoso Webb, B.S.

Project Role: Graduate Student

Nearest person months: 12 months

Contribution: Ms. Webb is my graduate student. She performed all microbiota studies and assists with the mouse model of IBD.

Funding Support: DOD and TTUHSC/State Account

## **8. Special Reporting Requirements**

Nothing to report

## **9. Appendix Items**

# Appendix Item 1

**In Press: *Inflammatory Bowel Diseases*, 2017**

## **Differential Susceptibility to T Cell-Induced Colitis in Mice: Role of the Intestinal Microbiota**

Cynthia Reinoso Webb, PhD., M(ASCP)<sup>CM\*1</sup>, Hendrik den Bakker, PhD.<sup>\*2</sup>, Iurii Kobozev, PhD.<sup>1</sup>, Yava Jones-Hall, DVM, PhD.<sup>3</sup>, Kameswara Rao Kottapalli, PhD.<sup>4</sup>, Dmitry Ostanin, PhD.<sup>5</sup>, Kathryn L. Furr, M.S.<sup>1</sup>, Qinghui Mu, B.S.<sup>6</sup>, Xin M. Luo, PhD.<sup>6</sup> and Matthew B. Grisham, PhD.<sup>1</sup>

\*Both authors contributed equally to the preparation of this manuscript

<sup>1</sup>Department of Immunology and Molecular Microbiology  
Texas Tech University Health Sciences Center  
Lubbock, TX 79430

<sup>2</sup>Center for Food Safety  
University of Georgia  
Athens, GA 30223

<sup>3</sup>Department of Comparative Pathobiology  
Purdue University College of Veterinary Medicine  
West Lafayette, IN 47907

<sup>4</sup>Center for Biotechnology and Genomics  
Texas Tech University  
Lubbock, TX 79409

<sup>5</sup>Immunology Discovery  
Translational Research and Development  
Bristol Myers Squibb  
P.O. Box 4000  
Princeton, NJ 08543-4000

<sup>6</sup>Department of Biomedical Sciences and Pathobiology  
College of Veterinary Medicine, Virginia Tech  
Blacksburg, VA 24061

Running title: Microbiota and Induction of Chronic Gut Inflammation:  
A Tale of Two Cities

Key words: inflammatory bowel diseases, Crohn's disease, ulcerative colitis, microbiota, mouse models

## Conflicts of Interest and Source of Funding

None of the authors have any conflicts of interest

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Matthew Grisham, PhD: Work reported in this manuscript was supported by grants from the NIH (R01-DK091269), DOD (W81XWH-11-1-0666) and the Presidents' Collaborative Research Initiative.

Address all correspondence to:

Matthew B. Grisham, Ph.D.

Department of Immunology and Molecular Microbiology

Texas Tech University Health Sciences Center

3601 4th Street STOP 6591

Lubbock, Texas 79430-6591

Phone: 806-743-2598

email: [matthew.grisham@ttuhsc.edu](mailto:matthew.grisham@ttuhsc.edu)

## Abstract

One of the best-characterized mouse models of the inflammatory bowel diseases (IBD; Crohn's disease, ulcerative colitis) is the CD4<sup>+</sup>CD45RB<sup>high</sup> T cell transfer model of chronic colitis. Following our relocation to Texas Tech University Health Sciences Center (TTUHSC), we observed a dramatic reduction in the incidence of moderate-to-severe colitis from a 15-year historical average of 90% at LSU Health Sciences Center (LSUHSC) to <30% at TTUHSC. We hypothesized that differences in the commensal microbiota at the two institutions may account for the differences in susceptibility to T cell-induced colitis. Using bioinformatic analyses of 16S rRNA amplicon sequence data, we quantified and compared the major microbial populations in feces from healthy and colitic mice housed at the two institutions. We found that the bacterial composition differed greatly between mice housed at LSUHSC vs. TTUHSC. We identified several genera strongly associated with, and significantly over-represented in high responding RAG<sup>-/-</sup> mice housed at LSUHSC. In addition, we found that that colonization of healthy TTUHSC RAG<sup>-/-</sup> mice with feces obtained from healthy or colitic RAG<sup>-/-</sup> mice housed at LSUHSC transferred susceptibility to T cell-induced colitis such that the recipients developed chronic colitis with incidence and severity similar to mice generated at LSUHSC. Finally, we found that the treatment of mice with *preexisting colitis* with antibiotics remarkably attenuated disease. Taken together, our data demonstrate that specific microbial communities determine disease susceptibility and that manipulation of the intestinal microbiota alters the induction and/or perpetuation of chronic colitis.

## INTRODUCTION

Animal models of the inflammatory bowel diseases (IBD; Crohn's disease, ulcerative colitis) have been invaluable in advancing our understanding of the immunopathologic mechanisms responsible for chronic intestinal inflammation (1, 2). Following more than two decades of investigations using a variety of genetically-engineered, spontaneous and immune-manipulated mouse models of IBD, investigators have concluded that chronic intestinal inflammation results from a dysregulated immune response to intestinal microbiota in genetically susceptible mice (3). The apparent interaction among genetics, the immune system and intestinal microbiota is not surprising given the results from genome-wide association studies of patients with IBD (4). More than 160 different polymorphisms/susceptibility loci have been identified in patients with Crohn's disease (CD) or ulcerative colitis (UC) (4). In CD, several susceptibility loci are known to be important for recognition and/or elimination of intracellular bacteria including nucleotide-binding oligomerization domain 2 (*NOD2*), autophagy-related protein 16-1 (*ATG16L1*) and immunity-related GTPase M (*IRGM*) (4, 5). These genetic risk factors together with polymorphisms related to epithelial barrier function, as well as IL-10, IL-17 and IL-23 signaling suggest that CD may develop because of defects in response against microorganisms (4, 5). Indeed, the low concordance rates for CD (<40%) or UC (<20%) in genetically identical twins strongly suggest that certain environmental factors (e.g. intestinal microbiota) are important in the induction and perpetuation of chronic intestinal inflammation in genetically susceptible individuals (6, 7). Investigators have found in both mouse models of IBD, as well as in human CD and UC, major alterations in luminal and mucosa-associated microbial communities resulting in a situation called dysbiosis, strongly suggesting that intestinal bacteria play a major role in driving IBD in genetically susceptible mice or humans (8-10). Despite the intense interest in the role that dysbiosis may play in the immuno-pathogenesis of chronic intestinal inflammation, it is currently not clear whether dysbiosis is a cause or consequence of chronic tissue inflammation.

One of the best-characterized mouse models of IBD is the CD45RB<sup>high</sup> T cell transfer model of chronic colitis (11, 12). Adoptive transfer of naïve CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from healthy wild type (WT) mice into recombina-activating gene-1 or -2-deficient (RAG-1<sup>-/-</sup> or RAG-2<sup>-/-</sup>) recipients generates large numbers of disease-producing Th1 and Th17 effector cells with little or no production of regulatory T cells (Tregs) (11). Once generated, these colitogenic effector cells home to the colon as well as other tissues where they induce chronic and unrelenting colitis within 6-8 weeks post T cell transfer (11, 12). Due to the conversion of the naïve T-cells to disease-producing effector cells in the absence of Tregs, colitis will develop in most T cell-deficient recipients (e.g. SCID, RAG-1<sup>-/-</sup>, RAG-2<sup>-/-</sup>, athymic nude, TCRβ<sup>-/-</sup> × δ<sup>-/-</sup>) following T cell transfer (3, 11, 13). In addition, it is well known that intestinal bacteria are required for induction of disease in this as well several other mouse models of IBD (14, 15). The T cell transfer model has also proven to be particularly useful in understanding the critical role that Tregs play in suppressing the activation of naïve T cells by commensal intestinal bacteria (11, 16). A non-exhaustive search of PubMed identifies more than 280 studies that have appeared over the past two decades using this mouse model of IBD. Unlike other mouse models of chronic intestinal inflammation that develop spontaneous and in some cases, highly variable disease, the vast majority of published studies using the T cell transfer model consistently report similar incidence and severity of colitis. Surprisingly, there has been no metagenomic analysis of the colonic microbiome prior to and following induction of colitis in this model.

Following our relocation to Texas Tech University Health Sciences Center (TTUHSC), we witnessed a surprising change in the phenotype of our T cell transfer model of IBD. We observed a significant reduction in our 16-year historical incidence of moderate-to-severe colitis of ~90% at LSU Health Sciences Center (LSUHSC) (11) to a much more variable disease with an incidence of moderate-to-severe disease of ~30% at TTUHSC. This change in phenotype occurred despite the fact that animal vendor, housing conditions and T cell preparations were virtually identical at both institutions. While alterations in mouse model phenotype following changes in animal husbandry, institution and/or animal source have been appreciated for a number of years, it has only been relatively recent that investigators have begun to systematically define how and why these types of changes affect disease phenotype in mouse models of autoimmune and chronic inflammatory diseases (15, 17-19). For example, it is well known that the onset and severity of chronic colitis that develops spontaneously in IL-10<sup>-/-</sup> mice may be quite variable depending upon the strain, background and host microbial composition (20, 21). More recent studies by Yang and coworkers reported that when identical C57B/6JIL-10<sup>-/-</sup> mice were housed under specific pathogen-free conditions (SPF) at two different animal facilities within the same city, one group of mice responded to *Helicobacter hepaticus* infection with robust typhlocolitis, whereas the other group failed to develop significant gut inflammation (22). They concluded that differences in the microbiota were a major contributor to the susceptibility of developing colonic inflammation. A similar set of observations have been reported by another group using mice rendered deficient in the flagellin receptor toll-like receptor 5 (TLR5<sup>-/-</sup>) (23, 24). These investigators found that TLR5<sup>-/-</sup> mice develop a spontaneous and highly variable colitis with only 10% of the mutant mice exhibiting overt colitis. Furthermore, they demonstrated that the few mice that did develop chronic colitis displayed a dysbiosis that was characterized by enrichment of Proteobacteria when compared to their non-colitic TLR5<sup>-/-</sup> littermates. The vast majority of published studies using the T cell transfer model have reported much less variation with respect to incidence and severity of colonic inflammation than other spontaneous models of IBD. However, there are published studies demonstrating that adoptive transfer of CD45RB<sup>high</sup> T cells into lymphopenic recipients either fails to induce colitis or induces only mild-to-moderate colonic inflammation that can be enhanced by infection with certain pathobionts such as *Helicobacter hepaticus* (13, 25-30).

The change in phenotype coupled to the lack of any detailed microbial data in this model prompted us to quantify and compare the colonic microbiota in healthy and colitic mice obtained from TTUHSC and LSUHSC and determine whether manipulation of colonic microbiota may alter the incidence, severity and/or perpetuation of chronic colitis at our current institution.

## MATERIALS AND METHODS

### *Animals and housing*

All animal experiments were conducted according to the protocols approved by the Texas Tech Health Sciences Center Institutional Animal Care and Use committee (IACUC). Male C57Bl/6J recombinaise activating gene-1 deficient (RAG-1<sup>-/-</sup>) mice, as well as wild type C57Bl/6J (WT) mice were obtained from the Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age and were housed for 2 weeks in the animal care facilities associated with TTUHSC and LSUHSC in standard microisolator cages (see below) at five mice per cage in standard microisolator conditions. Freshly collected feces from mice housed at LSUHSC were provided by our collaborator (Dr. Dmitry V. Ostanin). Both facilities maintain animals in an environment that is free of specified microbial agents that could interfere with the research carried out at the institutions and maintained on a 6am-6pm light cycle. Both animal facilities maintain sentinel testing programs that screen mice semi-annually for mouse hepatitis virus, mouse parvovirus (MPV1, MPV2, MPV3), minute virus of mice, mouse neurovirus, Theiler's murine encephalomyelitis virus, mouse rotavirus, Sendai virus, Mycoplasma pulmonis, pneumonia virus of mice, reovirus 3, Lymphocytic choriomeningitis virus, Ectromelia virus and ectoparasites. All animals in our study were found to be free of the micro-organisms mentioned above for both institutions. All animals housed at TTUHSC were maintained in microisolator housing in standard size cages (12 x 6.25 inches) with a Reemay® filter (spunbonded polyester nonwovens, Farmingdale, NJ) ventilated on a Tecniplast® vent rack (Varese, Italy) connected to the Tecniplast® Smart Flow air circulation system. Mice housed at LSUHSC were maintained in an identical manner except that Allentown air circulation system was used. The rooms and each individual cage at both institutions were subjected to positive pressure relative to the outside environment to prevent microbial contamination. All cages at both institutions were sterilized and furnished with wood chip bedding (7090 Sani-Chips, Harlan® Laboratories Inc. Indianapolis, IN) and cotton material for nest construction. Animals maintained at TTUHSC were provided irradiated ProLab® Isopro® RMH 3000 (LabDiet St. Louis, MO) rodent chow and non-acidified tap water *ad libitum* whereas mice housed at LSUHSC were given Teklad (Envigo) 7012 LM-485 rodent chow (Envigo, City) and non-acidified tap water *ad libitum* (Table S1).

### *Induction of Chronic Colitis*

Because mice from the same cage share their microbiota via coprophagy, we reasoned that multiple rounds of mixed co-housing would minimize these potential “cage effects” for our *in vivo* experiments. Thus, mice were subjected to a total of four rounds of mixed co-housing prior to their randomization into the different treatment groups. Briefly, mice were housed four to a cage upon their arrival from the vendor. Following 2-3 days of housing, mice underwent an additional three rounds of mixed co-housing over the course of 7-10 days such that all mice would possess a similar microbiota. Randomization of mice into the different treatment groups was accomplished using the Stat Trek random number generator (<http://stattrek.com/statistics/random-number-generator.aspx>). This PC-based calculator uses a statistical algorithm to produce random numbers for each mouse in the study.

Chronic colitis was induced in RAG<sup>-/-</sup> recipients via adoptive transfer of naïve (CD4<sup>+</sup>CD45RB<sup>high</sup>) T cells using our well-established protocol (11). Briefly, spleens were removed from healthy male, C57Bl/6J WT mice immediately upon euthanasia and placed in FACS

buffer [PBS containing 4% fetal calf serum (FCS) (Atlanta Biologicals)] on ice. Spleens were gently ground between two frosted slides into a cell suspension and passed through a 70µm cell strainer (Falcon®, Corning, NY) using a 10 mL syringe with a 26G x ¾ needle (BD®, Franklin Lakes, NJ), CD4 cells were enriched by negative selection using Dynabeads® Untouched Mouse CD4 Cell kit (Life Technologies AS, Norway) according to manufacturer's instructions. CD4 cells were then stained with allophycocyanin (APC) rat anti-mouse CD4 and phycoerythrin (PE) rat anti-mouse CD45RB antibodies (BD Pharminigen™ Franklin Lakes, NJ). CD4<sup>+</sup> T cells were sorted using the BD FACSJAZZ™ cell sorter for the brightest 40% (CD45RB<sup>high</sup> cells) and dimmest 15% (CD45RB<sup>low</sup> cells). Male C57BL/6J RAG<sup>-/-</sup> recipients were then injected (*i.p.*, 0.5 mls) with 5 x 10<sup>5</sup> CD4<sup>+</sup>CD45RB<sup>low</sup> T cells to be used as controls and with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells to induce chronic colitis. For some studies, CD4<sup>+</sup>CD45RB<sup>high</sup> → RAG<sup>-/-</sup> mice were generated at LSUHSC and shipped to TTUHSC at 2 weeks following T cell transfer where they were housed for an additional 6 weeks.

### ***Fecal microbial transplant (FMT)***

In addition to the conventional model of T cell induced chronic colitis, we also wished to assess the effects of colonizing RAG<sup>-/-</sup> mice housed at TTUHSC with feces obtained from healthy RAG<sup>-/-</sup> mice or from CD4<sup>+</sup>CD45RB<sup>high</sup> → RAG<sup>-/-</sup> mice with active colitis (6-8 weeks post T cell transfer) that were generated at LSUHSC. Colonization of RAG<sup>-/-</sup> mice housed at TTUHSC with feces obtained from LSUHSC mice was accomplished by FMT using a minor modification of the method described by Markle et al. (31). Briefly, pellets were weighed, transferred into a sterile container, suspended in sterile room temperature water and gently dispersed using the PowerGen® 125 homogenizer (Fisher Scientific; Waltman, MA) to create a homogeneous suspension. Suspensions were then placed on ice. One, two hundred and fifty microliter (250µl) aliquot of fecal suspension was delivered to each mouse via gastric gavage using sterile 20G x 1.5" single use feeding needles (Cadence Science; Staunton, VA). Gastric gavage with fecal suspension was repeated 24 hours later such that each mouse received a total of 2.0 mg feces/g body weight via the two transfers.

### ***Antibiotic administration and/or fecal microbial transplatation***

Chronic colitis was induced as described above. Briefly, RAG<sup>-/-</sup> mice were colonized with feces obtained from colitic mice housed at LSUHSC followed by adoptive transfer of 5 x 10<sup>5</sup> CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. At four weeks post T cell transfer, mice were randomized into 4 groups (N=8 mice per group) that received no treatment (control group), an antibiotic cocktail (ABX), fecal microbial transplant (FMT) or both ABX and FMT (ABX+FMT). Previous studies have demonstrated that >80% of the mice develop chronic colitis at 4 weeks post T cell transfer (32). Mice in the ABX group received (*p.o.*, *ad libitum*) a cocktail of 1.125 g of Aspartame (Asp-Phe-methyl ester; Sigma-Aldrich), 0.15g of vancomycin (Sigma-Aldrich) and 0.3 g of neomycin (Sigma-Aldrich) in 300 ml of sterile water according to the method of Shen et. al (33). Briefly, mice received the ABX cocktail for 60 hours at which time the ABX cocktail was replaced with drinking water containing 10% polyethelyne glycol (PEG) which was administered (*p.o.*, *ad libitum*) to fasting mice for an additional 12 hours. Following PEG treatment, mice were placed on ABX-containing drinking water and allowed free access to food for an additional 7 weeks. The FMT group received five daily gavages followed by one weekly gavage for the remainder of the

experiment until the end of the experiment with feces from a healthy WT mouse. All FMT treatments were performed using fecal pellets obtained from one healthy WT mouse that was the same sex and age as treatment groups. The ABX+FMT group received the same ABX protocol as described above followed by the five daily and once weekly FMT protocol (same as FMT only group).

### ***Fecal and tissue collection and preparation***

For mice housed at TTUHSC, colonic fecal pellets were removed from the colons of each mouse immediately following euthanasia using aseptic technique. Briefly, colons were excised, opened longitudinally and fecal pellets removed and quick frozen in liquid nitrogen. All fecal samples were stored at -80°C. Colonic tissue was saved for histopathological evaluation and tissue leukocyte determinations (see below). For mice housed at LSUHSC, fresh fecal pellets were obtained from healthy RAG<sup>-/-</sup> mice or from CD4<sup>+</sup>CD45RB<sup>high</sup> → RAG<sup>-/-</sup> mice with active colitis (6-8 weeks post T cell transfer). Because of the nature of these inter-institutional studies using mice from ongoing studies at LSUHSC, collection of individual fecal samples from each animal was not possible. Thus, fresh fecal pellets from either healthy or colitic RAG<sup>-/-</sup> mice housed at LSUHSC were collected from approximately 10-12 mice from each group and placed into one of two sterile collection tubes for each group. The fecal pellets were immediately frozen in liquid nitrogen and stored at -80°C for shipment (on dry ice) to TTUHSC for further analyses. Microbial DNA was isolated from ~100 mg of fecal pellets from each mouse generated at TTUHSC and from 5 random samples of fecal pellets (~100 mg each) from the pooled healthy or colitic RAG<sup>-/-</sup> mice collected at LSUHSC. The MoBio PowerSoil® DNA Isolation Kit (Carlsbad, CA) and FastPrep® 24 bead beater (MP Biomedicals, Santa Ana, California) were used to extract microbial DNA from each fecal sample. Each sample was subjected to three rounds of bead beating for 60 seconds each at 4.0 m/s (resting one minute between rounds) in the PowerBead Tubes following the manufacturer's guidelines.

### ***Bacterial 16S rRNA Sequencing***

Fecal DNA was sequenced using an Illumina® Miseq desktop analyzer (Illumina®), San Diego, CA. We used dual index paired-end sequencing strategy and prepared the samples for high throughput sequencing using a two-step PCR approach according to the protocol supplied by Illumina® (Illumina 16S Metagenomic Sequencing Library Preparation). The variable region V3 and V4 of bacterial 16S rRNA gene was amplified using universal bacterial primer set 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') that contained Illumina adaptors (34). The PCR products were then verified on 1.5% agarose gel to test the integrity. Index PCR was performed using Nextera XT index kit v2 (Illumina®) in which a unique index for each sample was attached to the Illumina sequencing adapter on each end. Following PCR, the amplicon products were cleaned using AMPure XP beads (Beckman Coulter Inc.) following the manufacturer's instructions and eluted in 50 µL of 10mM Tris buffer (pH 8.5). The cleanup products were then quantified in triplicates on Qubit 3.0 fluorometer using dsDNA HS Assay kit (Invitrogen, Carlsbad, CA). DNA concentration in nM was calculated for each sample based on the average of triplicates of concentration (ng/µL) and an average library size of 630 bp. All the libraries were then normalized by diluting to 4 nM using 10 mM Tris (at pH 8.5). Pooling of libraries was performed by aliquoting 5µL each of 4 nM diluted library. The library

pool with unique indices was run on the Agilent 2200 tape station (Agilent Technologies, Santa Clara, CA) using D1000 Screen Tape (Agilent Technologies, Santa Clara, CA) following the manufacturer's instructions to get the final size of the library pool. The final library concentration was determined by fluorometric quantification using Qubit (Invitrogen). Sequencing was performed using the MiSeq Reagent Kit v3 (600-cycle) (Illumina®) and the cartridge and reagents were handled according to the manufacturer's instructions. The library was denatured using 0.2 N NaOH and diluted to 9.0 pM using pre-chilled HT1 buffer solution. Similarly, PhiX control libraries were denatured and diluted to 9.0 pM. To get a spike-in of ~10% for low diversity libraries, 60 µL of 9.0 pM PhiX library and 540 µL of 9.0 pM library were mixed. To ensure efficient template loading, the combined sample was heat denatured at 96°C for 2 minutes followed by chilling on ice-water bath for 5 minutes and loaded into the MiSeq reagent cartridge. A mean cluster density of 961 K/mm<sup>2</sup> was obtained for the library and 85% of clusters passed the quality filter. The samples were demultiplexed and FASTQ files were generated utilizing MiSeq reporter software (Illumina®).

### **Bioinformatics Analyses**

The 16S rRNA amplicon sequence data was analyzed using the Bioconductor (35) workflow described by Callahan et al. (36). All functions described in this section are part of the DADA2 package (37) unless mentioned otherwise. Briefly, reads derived from paired end libraries were quality trimmed and dereplicated, using the `fastqPairedFilter`, `derepFastq` functions. The `dada` function was then used to infer sequence variants for each member of the pairs and the `mergePairs` function was used to merge forward and reverse reads of the inferred variants. Putative chimeric sequences were filtered from the dataset using `removeBimeraDenovo` function and taxonomic identities were assigned to the inferred sequence variants using the `assignTaxonomy` function, which uses the RDP-classifier (38). Because the mouse microbiome can contain a large proportion of previously uncharacterized taxa (e.g., families like 'S24-7' (Bacteroidales) we created a custom 16S rRNA database based on sequences of strains characterized as part of the Mouse Intestinal Bacterial Collection (miBC) (39) and all available type strains stored in the Ribosomal Database Project (accessed August 2016).

Quantitative insights into microbial ecology (QIIME, version 1.8.0) pipeline was used to calculate the species richness and diversity indices (Shannon, phylogenetic and Chao1) in order to measure alpha diversity within the sample. Rarefactions were created for all diversity measures and richness assessments by 10 repeated iterations at a sub-sampling depth of 10 to 90,010 sequences. All the alpha diversity indices and richness estimates were normalized to a sampling depth of 1,000 to correct the differences between samples as a result of sampling depth. Pair-wise distances between microbial communities based on phylogenic relatedness of whole communities were calculated using UniFrac method (beta diversity between samples) (40). Indicator species analysis was performed to determine the indicative species of each group of samples using '*indicspecies*' function (41) in R (R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2016). Statistical analyses were performed in R (R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2016) using the PhyloSeq package (42), which uses ggplot2 (H. Wickham. 2009. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag NY) to plot graphs. Additionally, the R MSA package (43) was used for sequence alignment and the Phangorn package (44) was used for phylogenetic inference. Differential abundance was tested

using DeSeq2 (45). R scripts, the custom database and rds files containing the operational taxonomic units Operational taxonomic unit (OUT) tables are available on GitHub (<https://github.com/hcdenbakker/DADA2MouseMicrobiome>) and sequence data deposited at the NCBI Sequence Read Archive.

For taxonomic and indicator species analyses described in Table 2, Figure 9 and Supplemental Figure 5, 16S rRNA sequencing reads in fastq format were obtained by unzipping the fastq.gz files by gunzip command on linux server. Sequencing reads in fastq format were obtained by unzipping the fastq.gz files by gunzip command on linux server. Processing of the demultiplexed samples was performed using the QIIME pipeline developed at CBG. Assembling of the paired end reads of each sample was done using PEAR software. This was followed by quality trimming the assembled sequences to remove poor quality reads using split\_libraries\_fastq.py script with default parameters which included: truncation of any reads that has three consecutive low quality base calls and trimming of sequence to last high quality score position which was defined by q equals to three. Sequences that were not at least 75% of the expected amplicon length together with ambiguous base calls were removed from the data pool. The 16S rRNA gene sequences were clustered based on 97% similarity of the reads and OTUs (operational taxonomic units) were recognized against the subset Greengenes database (<http://greengenes.lbl.gov>) using UCLUST algorithm. Alignment of the sequences on PyNast aligner (46) and allocating representative sequence from each OTU to taxonomy against Greengenes 16S rRNA reference sequences (gg\_97\_otus\_aug\_13) was done. Removal of singleton from data pool was performed before taxonomy was assigned and further analyzed. Texas Tech University high computational resource, Hrothgar was used to accomplish this computational data analysis. (40). Indicator species analysis was performed to determine the indicative species of each group of samples using ‘*indicspecies*’ function (41) in R (R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2016).

### **Quantification of total bacterial load**

Total bacterial load in fecal samples was quantified using real-time PCR with domain-specific primers F340 and R514 (47). Briefly, using *Lactobacillus rhamnosus* (ATCC 7469) genomic DNA as a reference organism, a five-point standard curve was generated using iTaq Universal Supermixes (Bio-Rad, Hercules, CA) on an Applied Biosystems cycler (Foster City, CA) with the 7500 cycler program: one cycle at 95 °C for 5 mins, followed by 40 cycles of 94 °C for 15 s and 63 °C for 45 s (48).

### **Macroscopic and histopathologic scoring**

At eight weeks following transfer of naïve T-cells or when mice lost >15% of their original body weight, RAG<sup>-/-</sup> mice were euthanized and their colons were removed, cleaned of fecal material and scored for macroscopic evidence of colitis using our previously published scoring criteria (11). Normal appearing colons were assigned a score of 0, mild bowel wall thickening in the absence of visible hyperemia was assigned a score of 1, moderate bowel wall thickening and hyperemia was given a score of 2, severe bowel wall thickening with rigidity and marked hyperemia was assigned a score of 3, and severe bowel wall thickening with rigidity, hyperemia and colonic adhesions was given a score of 4. Colonic length and weight were also measured to calculate weight-to-length ratios which provide a quantitative index of inflammation (11). In addition to macroscopic inflammation, representative sections of the proximal, mid and distal

colon were fixed overnight in 10% PBS-formalin, embedded in paraffin, cut into 5  $\mu$ m sections and stained with hematoxylin and eosin (H&E). The degree of inflammation in cross sections of the colon was assessed by an experienced pathologist (Dr. Yava Jones-Hall) blinded to treatment allocation, as previously described (49). The severity of the leukocyte infiltrate in the mucosa was assessed as none, mild, moderate or severe with scores of 0-3, respectively; the distribution of leukocytes was denoted as present in the lamina propria only, extending to the submucosa, and extending to the serosa with scores of 1-3, respectively; the distribution of erosion/ulceration was assessed as none, focal, multifocal or diffuse with scores of 0-3, respectively; necrosis was assessed as none, mild, moderate or severe with scores of 0-3, respectively; goblet cell loss was assessed as none, focal, multifocal or diffuse with scores of 0-3, respectively. The *number* of crypt abscesses was quantified per 10 high power fields and categorized as 1-2, 3-4, or 4+ (scores of 0-3, respectively). A score of 0 was assigned for each criterion not noted. Total disease score ranges from 0 to a maximum of 18 points based upon summation of each assigned criterion. In addition, ten high power fields were evaluated and the infiltration of PMNs or mononuclear cells was assigned scores that ranged from 0-3 corresponding to no, mild, moderate or severe infiltration, respectively. All PMN and mononuclear scores were normalized to the overall severity of inflammation in the tissue.

### ***Statistical analysis of animal studies***

For all animal studies, results are expressed in  $\pm$ SEM. Statistical significance between two groups was assessed by using a 2 tailed student's unpaired *t* test. For multiple comparisons, analysis of variance (ANOVA) was done followed by Šidák post hoc test to compare different groups.

## RESULTS

### ***Differential susceptibility to T cell-induced colitis of RAG<sup>-/-</sup> mice housed at two different institutions***

Over the past two decades, a number of different laboratories from around the world have used the CD45RB<sup>high</sup> T cell transfer model of IBD to investigate the immuno-pathological and immuno-regulatory mechanisms responsible for induction and suppression of chronic gut inflammation driven by the intestinal microbiota (11-14). Indeed, we have a 16 year history of using this model of IBD at our former institution (LSUHSC) which consistently produced moderate-to-severe colitis in 85-90% of the reconstituted recipients (11). Following our relocation to TTUHSC, we observed surprising yet consistent differences in the incidence and severity of disease. [Figure 1A](#) illustrates the individual variability in the development of chronic colitis in mice generated at TTUHSC when compared to data generated by our collaborators at LSUHSC. We found that mice housed at TTUHSC developed chronic colitis that appeared to segregate into two distinct groups with only 27% (8 of 30 mice) of the mice developing moderate-to-severe disease (blinded histopathological scores >7; termed high responders) while 73% of the mice (22 of 30 mice) developed mild or no colitis (blinded histopathological scores <7; termed low responders) ([Figures 1A & B](#)). In contrast, the incidence and severity of disease in mice housed at LSUHSC was found to be much greater with 15 of 16 mice exhibiting severe disease (blinded histopathological scores >10) ([Figures 1A & B](#)). No colitis developed in either group of mice reconstituted with CD45RB<sup>low</sup> T cells.

Interestingly, if RAG<sup>-/-</sup> mice were reconstituted with CD45RB<sup>high</sup> T cells at LSUHSC, transported to TTUHSC at 2 weeks following T cell transfer and then housed at the TTUHSC animal facility for an additional 6 weeks, 80% of the mice developed moderate-to-severe colitis ([Figure S1](#)). These data appeared to suggest that the environment (i.e. microbiota) in mice housed at LSUHSC is much more conducive for induction of robust disease. These data, coupled to the incomplete penetrance of robust disease in mice generated at TTUHSC prompted us to quantify the intestinal microbial composition of healthy RAG<sup>-/-</sup> mice housed at the two institutions.

### ***Microbial composition of healthy RAG<sup>-/-</sup> mice housed at LSUHSC vs. TTUHSC***

In order to ascertain how the housing environment may affect the composition of colonic microbiota in mice housed at the two animal facilities, we analyzed the fecal microbiota of healthy RAG<sup>-/-</sup> mice housed in ventilated microisolator cages at LSUHSC and TTUHSC. Taxonomic analyses showed similar relative abundances of the five major phyla in both groups of mice with no significant differences between groups ([Figure S2A](#)). OUT-based measures of community richness and diversity (Observed species, Chao1 and Shannon) showed that samples from LSUHSC (with the exception of two outliers) were less heterogeneous in OTU richness than those obtained from TTUHSC mice ([Figure S2B](#)). Although not statistically significant, Chao1 and Shannon indices suggested a trend for higher OTU diversity for the LSUHSC samples when evenness of abundance of OTUs were taken into account ([Figure S2B](#)). These data may indicate that the microbial community in TTUHSC samples may be dominated by fewer but highly abundant taxa. In contrast, Principal Coordinate Analysis (PCoA) of mouse fecal microbiota that takes into consideration OTU composition and relative abundance of these OTUs within a community (weighted UniFrac), revealed a clear separation of the two groups indicating significant differences between the OTU composition and relative abundance of the microbiota

obtained from mice housed at LSUHSC vs TTUHSC (Figure 2A). Similarly, DESeq2 analysis revealed significant differences ( $< 0.01$  multiple comparison adjusted p-value) *within and between* groups at the genus level, including differences in strain and/or species that may otherwise be missed when looking at higher taxonomic classifications. For example, we observed multiple OTU expression within 7 of the 22 genera significantly and differentially expressed in feces obtained from LSUHSC vs. TTUHSC mice suggesting the presence of different strains and/or species within these genera (Figure 2B). The vast majority of differentially expressed OTUs in feces from LSUHSC mice were associated with Firmicutes with fewer genera assigned to Bacteroidetes, Actinobacteria and Teneicutes (Figure 2B). Some of the most overrepresented genera associated with LSUHSC mice ( $>10$  log2 fold change) were *Ruminococcus*, *Bifidobacterium*, *Clostridium sensu stricto*, *Alistipes*, *Anaeroplasma* and *Barnesiella*. In addition, DESeq2 analyses of microbiota of TTUHSC revealed only 5 genera that were differentially and significantly overrepresented albeit at lower relative abundances in TTUHSC vs. LSUHSC mice including *Extibacter*, *Dorea*, *Flavonifactor*, *Blautia* and *Intestimonas* (Figure 2B). For reference, a log2 fold change of 10 represents a change of  $2^{10}$  or a 1,024 change in OTU expression.

### **Microbial composition of RAG<sup>-/-</sup> mice following induction of chronic colitis at LSUHSC vs. TTUHSC**

In order to identify microbial communities that may be differentially represented in colitic mice housed at the two institutions, we quantified and compared the 16S rRNA amplicon sequence data obtained from healthy vs. colitic mice housed at the two institutions. As we noted above, 93% of the mice generated and housed at LSUHSC developed severe colitis (histopathological scores  $>10$ ). Thus, we first wished to quantify and compare the fecal bacterial communities obtained from healthy vs. colitic mice housed at LSUHSC. We observed an expansion of the phyla Verrucomicrobia with a concurrent contraction of Firmicutes and Bacteroidetes in colitic mice when compared to healthy RAG<sup>-/-</sup> animals housed at LSUHSC (Figure 3A). In contrast to other animal and human studies, we did not observe significant expansion of Proteobacteria. Using DeSeq2 analysis to ascertain whether specific genera were differentially represented in the two groups, we found numerous genera whose relative abundance were significantly overrepresented in the mice with active colitis when compared to healthy RAG<sup>-/-</sup> mice. Of the 31 genera that were differentially and significantly overrepresented in the colitic vs. healthy group, *Murimonas*, *Muribaculum*, *Blautia*, *Clostridium sensu stricto*, *Bacteroides* and *Akkermansia* were the six most abundant genera in the colitic group ( $>10$  log2 fold change) (Figure 3B). Of note, 9 of the 31 OTUs that were differentially overexpressed in LSUHSC colitic vs. healthy mice, contained multiple OTUs within each genera suggesting different species and/or strains. Interestingly, we observed only one genus in the phyla Proteobacteria (*Desulfovibrio*) that was differentially overexpressed in the colitic group albeit at a lower relative abundance than the six, most prevalent genera (Figure 3B). In fact, the large majority of the differentially expressed genera in colitic LSUHSC mice were associated with the phyla Firmicutes.

Using the same approach, we next wished to quantify and compare the fecal microbial communities in the TTUHSC colitic mice (i.e. *high responders*) with those of healthy RAG<sup>-/-</sup> mice housed at TTUHSC. We observed no significant differences between the 5 major phyla of high responders vs. healthy RAG<sup>-/-</sup> mice (Figure 4A). Again, we failed to observe the presence of Proteobacteria in the colitic mice. DeSeq2 analysis did reveal significant increases in expression

of 15 OTUs (genera) from high responders with *Ruminococcus*, *Bifidobacterium*, *Barnesiella*, *Hydrogenoanaerobacterium*, *Clostridium XIVa*, *Longicella* and *Alistipes* representing the 7 most abundant genera in the high responder group ( $>7.5$  log<sub>2</sub> fold change) (Figure 4B). As we observed previously, the large majority of the differentially expressed genera in the high responders were members of the phyla Firmicutes.

We next wished to compare the fecal bacterial communities obtained from TTUHSC high vs. low responders. We found that  $\alpha$  diversity largely overlapped suggesting no major differences in phylogenetic composition and taxon abundance between these two groups (not shown). Despite the lack of clear differences in  $\alpha$ - and  $\beta$ - diversity, we observed a significant increase in the relative abundance of Bacteroidetes (49.1% vs. 26.1%;  $p < 0.01$ ) in high vs. low responders, respectively whereas the relative abundance of Verrucomicrobia in the low responder group was found to be significantly higher vs. the high responder group (40.3% vs. 6.8%;  $p < 0.01$ ) (Figure S3A). When we tested for the presence of differentially expressed OTUs between the two groups, we observed 8 different genera from two different phyla (i.e. Firmicutes and Bacteroidetes) that were significantly overrepresented in the high responder group including *Longicella*, *Blautia*, *Roseburia*, *Dorea*, *Acutalibacter*, *Hydrogenoanaerobacterium*, *Bifidobacterium*, *Erysipelotrichaceae incertae sedis*; only *Akkermansia* and *Oscilibacter* were overrepresented in the low responder group (Figure S3B). Two of the genera overrepresented in the high responder group (*Hydrogenoanaerobacterium*, *Bifidobacterium*) were significantly increased when compared to healthy RAG<sup>-/-</sup> mice (Figure S3B).

Given the histopathologic similarity between LSUHSC colitic mice and the TTUHSC *high responders*, we wished to ascertain the similarities and differences in the colonic microbial communities in these two groups. Principal coordinate analysis using weighted unifrac revealed a clear separation of both groups demonstrating a significant difference in their phylogenetic composition and taxon abundance (Figure 5A). We found that while both groups of mice had similar abundances of Bacteroidetes, Firmicutes, Tenericutes and Actinobacteria, the relative abundance of Verrucomicrobia was significantly increased in colitic LSUHSC mice vs. TTUHSC *high responders* (Figure S3A and Figure 3A). Furthermore, we observed significant differences in the expression of OTUs between the two groups. For example, colitic mice generated at LSUHSC contained at least 25 different genera that were differentially and significantly overexpressed when compared to TTUHSC high responders (Figure 5B). The vast majority of these overrepresented genera were associated with Firmicutes with many fewer genera assigned to Bacteroidetes and Verrucomicrobia. Some of the most overrepresented genera associated with the colitic LSUHSC group ( $>10$  log<sub>2</sub> fold change) were *Anaerostipes*, *Lactobacillus*, *Murimonas*, *Bacteroides*, *Clostridium sensu stricto*, *Turicibacter*, *Clostridium XVIII* and *Akkermansia* (Figure 5B). *Syntrophococcus* was the only genera that was significantly overrepresented in TTUHSC-*high responders*. In addition, we observed multiple OTU expression within 11 of the 31 genera, again suggesting the presence of different strains and/or species within these genera.

### **Transmission of chronic colitis via fecal microbial transplantation**

Data presented in the preceding section clearly identified major differences in the colonic bacterial communities of mice housed at LSUHSC versus TTUHSC. Therefore, we next wished to determine whether colonization of RAG<sup>-/-</sup> mice housed at TTUHSC with feces obtained from healthy or colitic RAG<sup>-/-</sup> mice housed at LSUHSC would alter the onset and/or severity of chronic

colitis induced by the adoptive transfer of naïve T cells. Mice that received colitic RAG<sup>-/-</sup> feces exhibited early clinical signs of disease including loose stools with occasional occult blood, kyphosis, piloerection and inactivity starting at 3 weeks post T cell transfer. Indeed, 3 of the 8 mice in this group died unexpectedly within the first 3 weeks following T cell transfer (Figure 6A). In contrast, mice that received fecal gavage from healthy RAG<sup>-/-</sup> mice began to develop clinical signs of disease beginning 4-5 weeks post T cell transfer with all 8 mice in the healthy gavage group surviving the entire 8 week observation period (Figure 6A). Upon euthanasia, most of the RAG<sup>-/-</sup> mice that were colonized with feces from healthy donors (5 of 8 mice) exhibited macroscopic signs of moderate-to-severe colitis including bowel wall thickening, rigidity and mild hyperemia as well as increased colon weight/length ratios (Figures 6B & C). In contrast, all 5 of the surviving mice that received *colitic gavage* exhibited macroscopic evidence of moderate-to-severe disease such as bowel wall thickening, rigidity and colonic hyperemia as well as increased colon weight/length ratios (Figures 6B & C). Although the mean macroscopic scores and weight/length ratios for mice that received colitic gavage tended to be higher than those of mice that received healthy fecal gavage, the differences were not significantly different.

Blinded histopathological analyses of these two groups of mice revealed that 71% (6 of 8 mice) of the mice that received healthy fecal gavage and 100% (5 of 5 mice) of the surviving mice that received colitic fecal gavage had moderate-to-severe disease (scores >7; Figure 7A and B). Mean histopathological scores for healthy vs. colitic gavage mice were 9.8±1.3 and 12.6±0.7, respectively. This contrasts greatly with data presented in Figure 1 showing that only 27% (8 of 30 mice) of TTUHSC RAG<sup>-/-</sup> mice that received T cells (but no fecal gavage) exhibited moderate-to-severe disease. Blinded histopathological analysis of colons from mice gavaged with either healthy or colitic feces exhibited transmural inflammation with similar numbers of leukocyte infiltration (T cells, PMNs, macrophages) into the mucosa, submucosa and muscle layer. Goblet cell dropout, epithelial erosions and crypt abscesses were observed to a greater extent in the mice gavaged with colitic feces.

The similar degree of colonic inflammation in the two groups prompted us to quantify and compare the fecal microbiome in RAG<sup>-/-</sup> mice that were colonized with microbiota derived from healthy or colitic feces prior to engraftment with T cells. Alpha and beta diversity analyses failed to identify any significant differences between the two groups (data not shown). Furthermore, we did not observe any significant differences in the relative abundance of the major phyla between the healthy vs. colitic gavage mice, nor did we observed significant differences in the total bacterial load between the two groups (Figures S4A). However, DESeq 2 analysis revealed major differences *within and between* certain genera in the two different groups. We observed increased expression of different OTUs *within* the genera *Muribaculum*, *Clostrium XIVa*, *Eubacterium*, *Ruminococcus*, *Blautia*, *Murimonas*, *Anaerostipes* and *Bacteroides* in colitic gavage vs. healthy gavage groups (Figure S4B). In addition, we identified several genera that were significantly overrepresented in the colitic gavage microbiome with *Aneoroplasma*, *Ruminococcus*, *Murimonas*, *Anaerostipes*, *Bacteroides*, *Turicibacter* and *Dorea* representing the most abundant genera (>10 log<sub>2</sub> fold change) in this group (Figure S4B). We did not observe any genera that were uniquely over-represented in the healthy gavage group. As with all other comparisons, most of the differentially overrepresented genera in either group were members of the phyla Firmicutes. We noticed a trend for a smaller bacterial load in mice that received colitic vs. healthy gavage, however these data were not statistically significant (Figure S4C)

In order to determine whether the increase mortality in the colitic gavage mice was due to the introduction of pathogenic bacteria present in feces obtained from colitic LSUHSC mice, we colonized healthy WT or RAG<sup>-/-</sup> mice housed at TTUHSC with colitic feces obtained from LSUHSC. In the absence of T cell transfer, neither group of mice developed any macroscopic or histopathological evidence of colitis at 8-10 weeks post fecal transfer suggesting that colitic feces do not contain pathogens that induce colitis (Figure 8).

### ***Therapeutic Efficacy of Antibiotic Administration and/or Fecal Microbial Transplantation to Treat Preexisting Colitis***

It is well known that commensal microbiota are required for induction of chronic colitis in a number of mouse models of chronic colitis. Indeed, a number of studies have demonstrated that antibiotic treatment or germ-free conditions dramatically attenuates or eliminates the *development* of colonic inflammation in several mouse models of IBD, including the T cell transfer model of chronic colitis (14, 15, 50, 51). Furthermore, fecal microbial transplantation (FMT) has been identified as a promising therapeutic for diseases where the intestinal microbiota play a role in the induction and/or perpetuation of intestinal inflammation. Nieuwdorp and coworkers demonstrated that the transplant of fecal microbiota obtained from healthy donors into patients with recurrent *Clostridium difficile*-induced colitis, essentially cures these individuals with a rate of >90% compared to a rate of <35% with vancomycin. These data suggest that re-establishing a “healthy” microbiota may be a more potent and longer lasting treatment for inflammatory diseases such as IBD (52). No studies have been reported evaluating the therapeutic efficacy of antibiotic administration and/or FMT to treat ***preexisting*** colitis in our mouse model of IBD. Therefore, we initiated a series of studies to evaluate the therapeutic effects of antibiotic (ABX) administration, FMT administration or both treatments in mice with established disease. Colitis was induced in mice via gastric gavage with feces obtained from colitic RAG<sup>-/-</sup> mice housed at LSUHSC followed by adoptive transfer of naïve T cells. All treatments started at 4 weeks post T cell transfer based on our previous studies demonstrating that >80% of the mice develop active colitis at 4 weeks following T cell transfer (32).

We found that ABX and ABX+FMT, but not FMT alone were effective at reducing the increases in colon weight/length ratios in untreated mice (Figure S5A). Blinded histopathological analysis revealed moderate-to-severe disease in the control and FMT groups whereas colonic inflammation in mice treated with ABX or ABX+FMT was significantly attenuated (Figure 9A). Colons from the control group showed focal, moderate-to-severe PMN and mononuclear leukocyte infiltration extending into the serosa whereas colons from the FMT group revealed focal PMN and mononuclear infiltrate that extended to the submucosa. Epithelial cell necrosis, crypt abscesses and goblet cell drop out appeared more prominent in the control group (Figure 9B). Treatment with ABX dramatically and significantly reduced the severity of colitis when compared to the control group (Figure 9A and B). Microscopic examination of colons from the ABX group revealed little or no infiltration of PMNs with only two animals exhibiting mild infiltration of monocytes that was restricted to the lamina propria. In addition, these mice did not show necrosis, crypt abscesses, goblet cell loss or erosions. Blinded histopathological analysis of colons from mice in the ABX+FMT showed little or no infiltration of PMNs but moderate infiltration of monocytes distributed into the lamina propria (Figure 9B). Similar to the ABX treated group, colons from ABX+FMT mice did not show necrosis, crypt abscesses, goblet cell loss or erosion (Figure 9B).

Surprisingly, FMT treatment failed to attenuate chronic colitis when compared to their untreated controls.

We next wished to determine how the different treatment groups affected the major bacterial populations in each animal. While total bacterial load was reduced by 100 fold in response to ABX treatment (data now shown), this difference was not significant. We did however, observe a dramatic increase in relative abundance of Proteobacteria with a concomitant disappearance in Bacteroidetes in the ABX treated group ([Figure 9C](#)). We also observed a modest but not significant expansion of the phyla Verrucomicrobia in the control, FMT and ABX+FMT groups ([Figure 9C](#)). Principle coordinate analysis revealed that the ABX treated group was the only group that was significantly different from the control group ([Figure S5B](#)). Thus, we wished to determine which bacterial species were unique to ABX treated when compared to the control group by performing the indicator species analysis (ISA). ISA identifies bacterial OTUs that are significantly associated with one group when compared to the other by taking into consideration fidelity (exclusivity) and relative abundance of the organism (41). When we compared both groups, we identified several indicator species that were significantly ( $p < 0.05$ ) and uniquely associated with the control and ABX treated groups. Not surprisingly, many of the genera uniquely overrepresented in the ABX treatment group were members of the phyla Proteobacteria, while those uniquely overrepresented in the control group belonged to the phyla Firmicutes ([Table 2](#)).

## DISCUSSION

Mouse models of IBD have been used extensively for more than 25 years to gain a better understanding of the immuno-pathogenesis of IBD, as well as to identify and evaluate new therapeutic strategies that may be used to treat individuals with these chronic inflammatory disorders. Unfortunately, the penetrance/incidence and severity of disease in several different mouse models of IBD may be quite variable, presenting investigators with significant challenges in experimental design due to the inability to control the onset and uniformity of disease. This is not surprising given the growing body of evidence demonstrating that the intestinal microbiota within the same strain of mouse may vary greatly depending upon the animal vendor, the vendor location and even the room where the animals are housed within the same facility (53-55). Given our experience with differences in disease incidence and severity at two different institutions, coupled to the lack of detailed microbiome analyses in the T cell transfer model of IBD, we quantified and compared the major microbial populations in feces obtained from healthy and colitic mice housed at the two institutions. We identified several genera associated with the colonic microbiota obtained from the highly susceptible RAG<sup>-/-</sup> mice housed at LSUHSC that were significantly over-represented compared to that obtained from the more resistant TTUHSC RAG<sup>-/-</sup> mice. In addition, we found that severe disease developed in RAG<sup>-/-</sup> mice at TTUHSC provided that T cell transfer occurred and animals were housed at the LSUHSC animal facility for an initial 2 week period prior to transfer to TTUHSC. Furthermore, we observed that colonization of TTUHSC RAG<sup>-/-</sup> mice with fecal microbiota from healthy LSUHSC RAG<sup>-/-</sup> mice could transfer disease susceptibility such that the incidence and severity of colonic inflammation approximated that observed in the highly susceptible LSUHSC mice following T cell transfer. Finally, we found that ABX but not FMT treatment significantly attenuated preexisting disease. We discuss our novel findings of this study in the context of disease pathogenesis.

Although several studies have characterized the intestinal microbiota in different mouse models of IBD with varying susceptibilities to spontaneous colitis, no studies have reported alterations in the colonic microbiome in the T cell transfer model of chronic colitis. In the current study, we observed significant increases in abundance of a number of specific genera within the colonic microbiota of LSUHSC vs. TTUHSC mice. We found a total of 22 different genera that were significantly overrepresented in the highly susceptible LSUHSC RAG<sup>-/-</sup> mice with *Ruminococcus*, *Bifidobacterium*, *Clostridia sensu stricto*, *Alistipes*, *Anaeroplasma* and *Barbesiella* representing the genera with the highest abundance in this group (Figure 2B). Although we did not observe significant differences in the overall abundance of *Muribaculum* between the two groups, DESeq2 analysis revealed significantly more strains and/or species within this genus in feces collected from healthy LSUHSC RAG<sup>-/-</sup> vs. TTUHSC RAG<sup>-/-</sup> mice (8 vs. 4 strains/species, respectively; Figure 2B). It is not apparent at the current time which of the different genera are required for induction of colitis. There are however, a few reports demonstrating that certain mucolytic bacteria from the genus *Ruminococcus* (e.g. *R. gnavus* and *R. torques*) are increased in the non-inflamed and inflamed mucosa of patients with IBD (56). We are currently attempting to identify the specific species that are overrepresented in the highly susceptible LSUHSC animals.

In addition to the unique genera that colonize healthy RAG<sup>-/-</sup> mice from LSUHSC, we observed 4 genera that were significantly overrepresented in the TTUHSC mice when compared to LSUHSC RAG<sup>-/-</sup> animals. These genera include *Extibacter*, *Dorea*, *Flavonifractor*, *Blautia* and *Intestimonas* (Figure 2B). Very little is known about the relationship between these genera and

susceptibility to gut inflammation; however, *Blautia* has recently been identified as a major butyrate (and/or acetate) producer (57). In fact, *Blautia spp.* are among the most abundant bacteria within the gastrointestinal tract comprising between 2.5% and 16% of the total microbiota (58). In addition, *Blautia* has been suggested to possess anti-inflammatory activity as it has been shown to be associated with reduced risk of graft-versus-host-disease, as well as improved outcomes in colorectal cancer, inflammatory pouchitis and liver cirrhosis (59). It is intriguing to speculate that future studies may reveal the immunosuppressive/protective properties of specific species within these genera which may be useful in suppressing intestinal inflammation.

The reasons for the differences in composition of the commensal microbiota between the two groups of RAG<sup>-/-</sup> mice have not been clearly defined at the present time. It is well-documented that a number of factors may affect the composition of the intestinal microbiota in rodents including diet, genetics/strain, age, sex, pH of the drinking water and stress (55, 60, 61). Given that all except for one of these variables, as well as housing conditions are very similar, if not identical at the two institutions, we believe that differences in diet may have had a major effect on bacterial composition and consequent induction of chronic gut inflammation. Indeed, retrospective examination of the rodent chow composition used at the two institutions reveals a few major differences (Table S1). One difference is the addition of porcine animal fat that is preserved with butylated hydroxyanisole (BHA) in the TTUHSC chow. This lipid-soluble compound is well known to be a potent antioxidant and free radical scavenger. It is used widely in the human and animal food industry to reduce lipid peroxidation limiting the rancidification of polyunsaturated fatty acids. It is quite possible that addition of this xenobiotic in combination with porcine fat alters the intestinal microbiome from one that promotes T cell activation and expansion of disease-producing effector cells (e.g. Th1 and Th17 cells) to a commensal community that is unfavorable for induction of disease. An equally intriguing hypothesis is that BHA/porcine fat does not affect the initial T cell responses to commensal bacterial antigens but suppresses the subsequent inflammatory cascade including inhibition of T cell and/or myeloid cell trafficking to the gut, as well as suppression of inflammatory cytokine production by T cells, monocytes and granulocytes (i.e. PMNs). It is well-known that antioxidants, including BHA, possess potent anti-inflammatory activity *in vitro* and *in vivo* (62, 63). Another major difference between the two diets is the addition of fish meal to the TTUHSC chow (Table S1). However, it is not clear how addition of this protein source might affect microbial communities and/or the inflammatory process.

Another novel observation made in the current study was that robust disease does in fact develop in RAG<sup>-/-</sup> mice housed at TTUHSC, provided that T cell transfer occurs and animals are housed at the LSUHSC animal facility for an initial 2 week period prior to transfer to TTUHSC (Figure S1). These studies suggest that once the initial immunological interactions occur between naïve CD4<sup>+</sup> T cells and “appropriate” microbial antigens, progression of chronic disease will continue over the ensuing 6 weeks irrespective of the animal care facility. How this occurs is only a matter of speculation; however, one could envision a scenario where low-grade, intestinal inflammation is induced during the first two weeks following T cell transfer at LSUHSC thereby creating a dysbiotic microbiota (5, 64). This subclinical, inflammation-induced dysbiosis would continue unabated over the ensuing weeks resulting in the expansion of pathobionts that are ultimately responsible for the induction of chronic colitis (64). Inflammation-induced dysbiosis would not only initiate disease in our lymphopenic RAG<sup>-/-</sup> recipients, but it would also perpetuate colitis irrespective of the animal care facility. If this scenario is correct, then one would predict

that colonization of healthy RAG<sup>-/-</sup> mice housed at TTUHSC with feces obtained from RAG<sup>-/-</sup> donors housed at LSUHSC would render these mice more susceptible to induction of chronic colitis. In fact, that is exactly what we observed (Figures 6 and 7). If, as Schaubeck et. al. suggest using the TNF<sup>ΔARE</sup> mouse model of chronic ileitis, that inflammation-induced dysbiosis may “cause transmission of disease to the susceptible host” (65), then we would predict that colonization of TTUHSC RAG<sup>-/-</sup> mice with feces obtained from colitic LSUHSC donors would produce robust disease following T cell transfer. These data would be consistent with the concept that time-dependent, inflammation-induced dysbiosis is a rate determining step in induction of disease.

One of the most consistent observations reported in patients with IBD and in some (but not all) mouse models of IBD is the expansion of Proteobacteria with a concomitant reduction in Firmicutes (64, 66). Therefore, we were surprised to find no significant expansion of Proteobacteria or its associated families (e.g. *Enterobacteriaceae*, *Helicobacteraceae*, etc) in colitic mice generated at LSUHSC or at TTUHSC (i.e. high responders) (Figures 3, 4, 5 and S3). On the contrary, we observed that the large majority of the top 5-7 most overrepresented genera in colitic mice generated at the two institutions were members of Firmicutes with substantially fewer genera associated with Bacteroidetes and Verrucimicrobia (Figures 3, 4, 5 and S3). In addition, we observed that the composition of the genera in the different groups of colitic mice were, in most cases, quite different with little overlap. At first glance, these data appear to be surprising since colitic mice at LSUHSC and TTUHSC (high responders) were generated using the same protocol. In fact, this is not the case. These novel and somewhat provocative data suggest that depending upon the initial microbial composition of the healthy mice, the components of the dysbiotic microbiota in colitic mice may be very different. Thus, the ability to identify specific genera and/or species may be much more difficult than originally thought. For example, we did observe a large and significant over abundance of two genera found within the phyla Bacteroidetes (e.g. *Muribaculum* and *Bacteroides*) in colitic LSUHSC mice (Figure 3). It has been reported that a member of Bacteriodes (e.g. *Bacteriodes thetaiotaomicron*) is capable of digesting mucin and inducing chronic colitis in antibiotic-treated, genetically-susceptible mice (67). However, these authors also noted that while *Enterobacteriaceae* were >100 fold enriched in spontaneous colitis in these same mice, monoassociation of antibiotic-treated mice with these facultative anaerobes did not induce chronic colitis in genetically susceptible animals (67). These data are similar to those reported by Perez-Munoz et. al. who found that development of spontaneous colitis in their genetically-engineered mouse model of IBD was associated with large and significant increases in abundance of *Lactobacillus* and *Clostridium* species (68). However, monoassociation of their germ free mice with either species failed to induce disease whereas colonization with *Bacteriodes thetaiotaomicron* induced robust colitis. Complicating the situation even more, Garret et. al. reported that although the relative abundance of two members of *Enterobacteriaceae* were increased and correlated well with the development of spontaneous colitis in their genetically-susceptible mice, they could only induce disease in germ free mice when *Enterobacteriaceae* were administered together with commensal bacteria (69).

Another novel observation we made in the current study was that the microbiota within the colons of LSUHSC colitic mice contain a large and significant expansion of the genera *Akkermansia* (Figure 3). The only known species of this genera is the mucin-degrading bacterium *Akkermansia muciniphilia*. This bacterium has been shown to be increased in experimental and

human IBD (70-72). Paradoxically, we also observed large and significant increases in abundance of this same bacterium in the feces of mice that develop little or no colitis at TTUHSC (low responders; [Figure S3](#)). Indeed, others have reported the protective effects of *Akkermansia muciniphila* in mice and humans (73). Although it is not clear how and why this bacterium can be overrepresented in feces of mice with active and quiescent disease, we hypothesize that different strains of *Akkermansia muciniphila* may affect disease outcome. It is also possible that *Akkermansia muciniphila* alone plays no role in disease pathogenesis and/or protection or it affects disease phenotype only in the presence of other bacterial communities. In addition, it is quite possible that the disease outcome differences may be related to the loss of low-abundance organisms. Although certain organisms could be present in a relatively low abundance, they could be metabolically very active and their metabolites could be detrimental or beneficial to the host and other microbial communities.

As mentioned previously, it is well-known that commensal bacteria are required for induction of chronic intestinal inflammation in several different mouse models of IBD. Virtually all studies implicating the intestinal microbiota in the pathogenesis of experimental IBD have used either prophylactic antibiotic ABX treatment or germ free mice. To our knowledge, no studies have been performed evaluating the therapeutic efficacy of ABX treatment in a more clinically-relevant situation where mice have preexisting colitis. Data obtained in the current study demonstrate, for the first time, that administration of broad spectrum ABX to mice with established disease remarkably attenuates colonic inflammation ([Figure 9](#)). In addition, while ABX-treated mice appeared to have many fewer bacteria than controls, we observed a significant expansion of Proteobacteria, as well as loss of Bacteroidetes, when compared to untreated controls ([Figure 9](#)). At first glance, these data appear to be counter intuitive as many human and some animal studies suggest that expansion of certain bacteria within Proteobacteria are strongly associated with disease. Although the reasons for this apparent disconnect are not known, it may be that one or more of the overrepresented genera following ABX treatment are protective or antiinflammatory in nature [e.g. *Lactobacillus* (phyla Firmicutes); [Table 1](#)]. Alternatively, the surviving members of Proteobacteria are not disease-producing pathobionts or simply cannot induce chronic colitis at their reduced numbers following ABX treatment. In addition, it may be that ABX treatment may itself have some sort of immunomodulatory effect independent of their antibiotic activity. Nevertheless, ABX treatment can be effective in attenuating distal bowel disease, it is currently used as an adjunctive therapy (74).

Another objective of the current study was to assess the therapeutic efficacy of FMT in mice with preexisting disease. As mentioned previously, a great deal of excitement has been generated following the report by Nieuwdorp et. al. in the New England Journal of Medicine demonstrating that FMT essentially cured (>90% efficacy) **recurrent** *Clostridium difficile*-induced colitis (52). We found that FMT administration alone did not attenuate colonic inflammation even when administered multiple times following the onset of disease ([Figure 9](#)). In some respects, these data are not terribly surprising given recent clinical reports demonstrating little or only modest effects of FMT in patients with active IBD (75). Although we observed that the combination of ABX+FMT significantly reduced colonic inflammation, protection was similar to ABX treatment alone suggesting that broad-spectrum antibiotics are much more effective than FMT in treating established disease ([Figure 9](#)).

When taken together, this study of the colonic microbial composition of mice with differential susceptibility to T cell-induced colitis should allow for the generation of testable hypotheses to ascertain which bacteria may be responsible for promoting inflammation or suppressing disease. While we were able to identify relatively few candidate genera that were differentially overrepresented in highly susceptible LSUHSC mice vs. those in the resistant TTUHSC mice, we recognize the limitations of our study. Data obtained in our current study as well as those obtained in numerous others by different laboratories are limited by the inability to definitively define the causive species and/or strains of bacteria in mouse models of chronic disease. To a large degree, this is due to limitations inherent in the methods used for the analyses of intestinal microbial communities. The bioinformatics platforms used by the large majority of investigators for 16S rRNA amplicon gene and metagenomics/transcriptome analyses are currently less than optimal. For example, most of the commonly used 16S rRNA analyses are incapable of providing phylogenetic data lower than the genus level (76, 77). In addition, databases for analyzing metagenomic and transcriptomic analyses are incomplete, thereby hindering our ability to gain insight into microbial structure and function (76, 77). Nevertheless, we have taken a major step forward in defining the role of intestinal bacteria in the pathogenesis of chronic gut inflammation.

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## Figure Legends

### **Figure. 1 Induction of chronic colitis in mice housed at two different institutions.**

**A)** Blinded histopathology scores of colons from mice at 6-8 weeks following transfer of either CD4<sup>+</sup>CD45RB<sup>low</sup> or CD4<sup>+</sup>CD45RB<sup>high</sup> T cells into RAG<sup>-/-</sup> mice housed at LSUHSC or TTUHSC. A total of 18 and 16 mice were used for the CD4<sup>+</sup>CD45RB<sup>low</sup> or CD4<sup>+</sup>CD45RB<sup>high</sup> T cell groups at LSUHSC, respectively whereas 4 and 30 mice were used for the CD4<sup>+</sup>CD45RB<sup>low</sup> or CD4<sup>+</sup>CD45RB<sup>high</sup> T cell groups at TTUHSC, respectively. **B)** Representative micrographs of colons from mice reconstituted with CD4<sup>+</sup>CD45RB<sup>low</sup> or CD4<sup>+</sup>CD45RB<sup>high</sup> T cells housed at the two institutions. Data are presented as the mean±SEM for the animals indicated.

**Figure 2. Principal coordinate and DESeq analyses of fecal microbiota from healthy RAG<sup>-/-</sup> mice housed at LSUHSC or TTUHSC.** **A).** Principal coordinate analysis using weighted UniFrac of fecal microbiota for healthy RAG<sup>-/-</sup> mice housed at the two institutions. Weighted UniFrac takes into consideration the OTU composition and relative abundance of the OTUs within a community. Each axis shows the percentage of variation that is present in the dataset, Axis1 being the largest representation of variability. LSUHSC and TTUHSC microbiota samples are shown as purple and red circles, respectively. Note the clear separation of the two groups indicating significant differences in the bacterial populations between the two institutions. **B).** Differential overexpression of bacterial OTUs (genera) in feces obtained from healthy RAG<sup>-/-</sup> mice housed at LSUHSC or TTUHSC. Using DESeq analysis, values >0 represent OTUs (genera) that are significantly overexpressed in healthy RAG<sup>-/-</sup> mice housed at TTUHSC whereas values <0 represent genera that are significantly overrepresented in healthy mice housed at LSUHSC. Multiple dots aligning within one genera represent different strains and/or species.

**Figure 3. Relative abundance and differential overexpression of fecal bacterial communities in healthy and colitic RAG<sup>-/-</sup> mice housed at LSUHSC.** **A).** Relative abundance of the six major phyla in five individual fecal samples each from healthy and colitic mice housed at LSUHSC. **B).** Differential overexpression of OTUs (genera) in feces from healthy and colitic mice housed at LSUHSC. Using DESeq analysis, values >0 represent OTUs (genera) that are significantly overexpressed in healthy RAG<sup>-/-</sup> mice whereas values <0 represent OTUs (genera) that are significantly overexpressed in colitic RAG<sup>-/-</sup> mice. Again, multiple dots aligning within one genera represent different strains and/or species.

**Figure 4. Fecal bacterial communities in healthy and colitic mice (high responders) housed at TTUHSC.** **A).** Relative abundance of the six major phyla in feces from healthy (N=5) and colitic RAG<sup>-/-</sup> mice (high responders; N=5) housed at TTUHSC. **B).** Differential overexpression of OTUs (genera) in feces from healthy and colitic (high responders) mice housed at TTUHSC. Using DESeq analysis, values >0 represent OTUs (genera) that are significantly overexpressed in colitic RAG<sup>-/-</sup> mice (high responders) whereas values <0 represent OTUs (genera) that are significantly overexpressed in healthy RAG<sup>-/-</sup> mice. Multiple dots aligning within one genera represent different strains and/or species.

**Figure 5. Principal coordinate and DESeq analyses of fecal microbiota from colitic RAG<sup>-/-</sup> mice housed at LSUHSC or TTUHSC.** **A).** Principal coordinate analysis using weighted UniFrac of fecal microbiota from colitic mice housed at the two institutions. LSUHSC and TTUHSC (high responder) samples (N=5) are shown as red and black circles, respectively. Note the clear separation of the two groups indicating significant differences in microbial populations between the two institutions (beta diversity). **B).** Differential overexpression of OTUs (genera) in feces from colitic mice housed at TTUHSC or LSUHSC. Values >0 represent OTUs (genera) that are significantly overexpressed in TTUHSC high responders whereas values <0 represent OTUs (genera) that are significantly overexpressed in colitic mice housed at LSUHSC.

**Figure 6. Chronic colitis develops in RAG<sup>-/-</sup> mice colonized with healthy or colitic feces prior to T cell transfer.** A total of 16 RAG<sup>-/-</sup> mice housed at TTUHSC were colonized (via gavage) with a suspension of feces obtained from either healthy or colitic RAG<sup>-/-</sup> mice housed at LSUHSC prior to T cell transfer (N=8 for each group; see methods section). **A)** Survival of mice gavaged with healthy (N=8 to start; all survived) or colitic (N=8 to start; 3 died) feces. **B)** Macroscopic colon scores at 8 weeks post T cell transfer. Macroscopic scores were based upon a 0-4 scoring system for gut thickness, rigidity, hyperemia and shortening (a score of 0 represents a healthy colon). **C)** Colonic weight-to-length ratios at 8 weeks post T cell transfer. A weight/length ratio of a healthy colon is indicated by green dashed line (~0.020). Data in Figures **B** and **C** were derived from the surviving 8 and 5 mice in the healthy and colitic gavage groups, respectively and are presented as the mean±SEM.

**Figure 7. Blinded histopathology scores of colons from mice colonized with healthy or colitic feces prior to T cell transfer.** A total of 16 RAG<sup>-/-</sup> mice housed at TTUHSC were colonized (via gavage) with a suspension of feces obtained from either healthy or colitic RAG<sup>-/-</sup> mice housed at LSUHSC prior to T cell transfer. All 8 mice in the healthy gavage group survived whereas only 5 animals in the colitic gavage group survived the eight week observation period. **A)** Blinded histopathology scores were determined at 6-8 weeks following T transfer. **B)** Representative micrographs of colons from mice in the different groups. Data from Figure 1A showing T cell-engrafted RAG<sup>-/-</sup> mice housed at TTUHSC (No gavage + T cell group; N=30) is included for comparison. Data are presented as the mean±SEM.

**Figure 8. Chronic colitis fails to develop in RAG<sup>-/-</sup> or wild type mice colonized with colitic feces.** RAG<sup>-/-</sup> (N=4) and wild type (WT; N=3) mice housed at TTUHSC were colonized with colitic feces (from LSUHSC) but were not engrafted with T cells. Data from Figure 7A showing histopathology scores for RAG<sup>-/-</sup> mice colonized with colitic feces and engrafted with T cells (N=5) is included for comparison. Data are presented as the mean±SEM.

**Figure 9. Therapeutic effects of antibiotic treatment and/or fecal microbiota transplant in mice with preexisting colitis.** Treatment with antibiotics (ABX; neomycin and vancomycin; N=7), fecal microbial transplant (FMT; N=8) or both ABX and FMT (N=5) began 4 weeks following T cell transfer as described in the Methods section. Seven mice were not treated and served as the control group. **A)** Blinded histopathology scores of colons for all treatment groups at 11 weeks post T cell transfer. Data are expressed as the mean±SEM. **B).** Representative

micrographs from each group. Note the extensive inflammatory infiltrate (blue circles) and goblet cell dropout in the control (untreated) and FMT groups. Scores in each micrograph (in white) represent the mean histopathology scores for that group. **C).** Relative abundance of the five major phyla in feces obtained from mice subjected to ABX and/or FMT treatment. Note the large expansion of Proteobacteria and major reductions of Bacteroidetes and Verrucomicrobia in mice treated with the ABX cocktail.

Figure 1

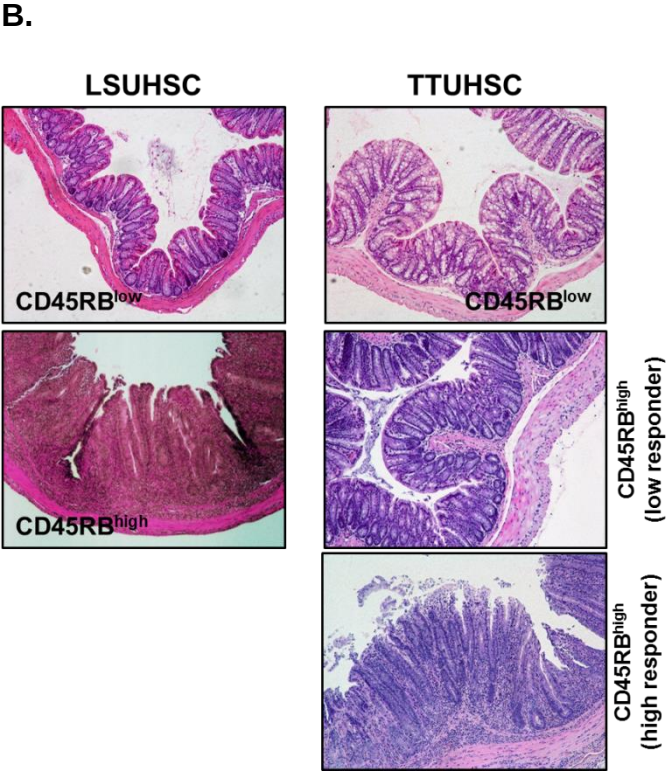
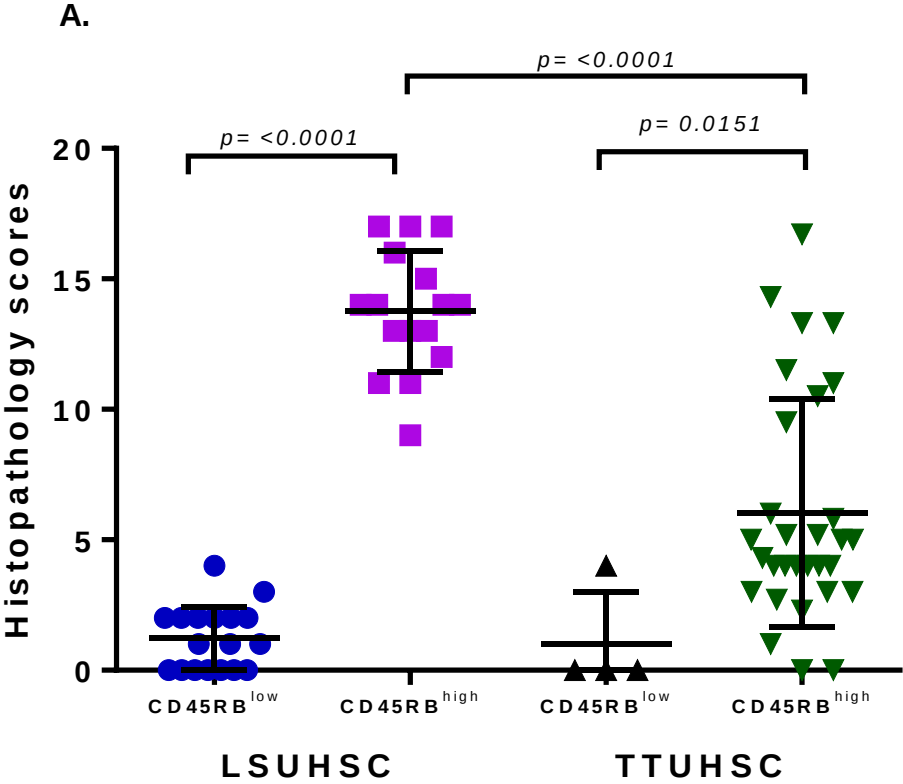


Figure 2

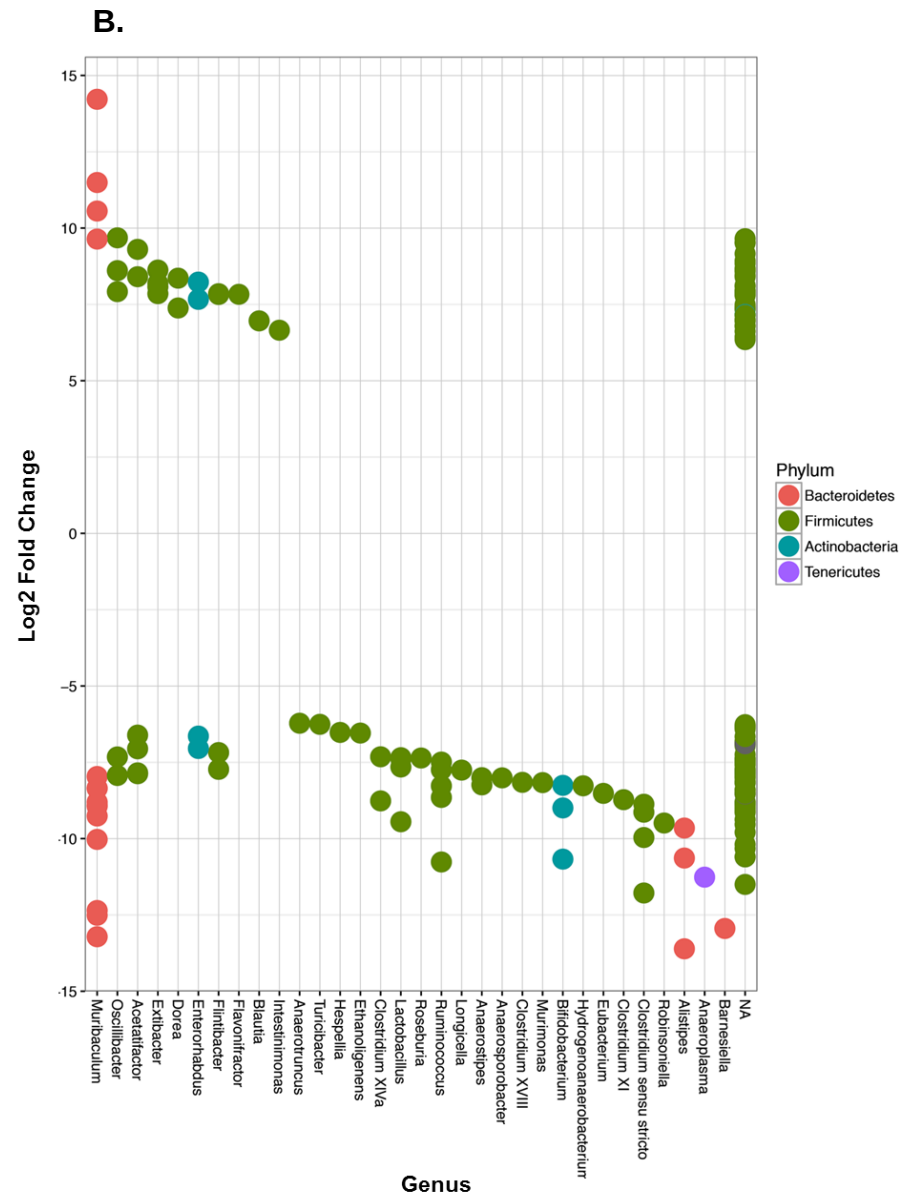
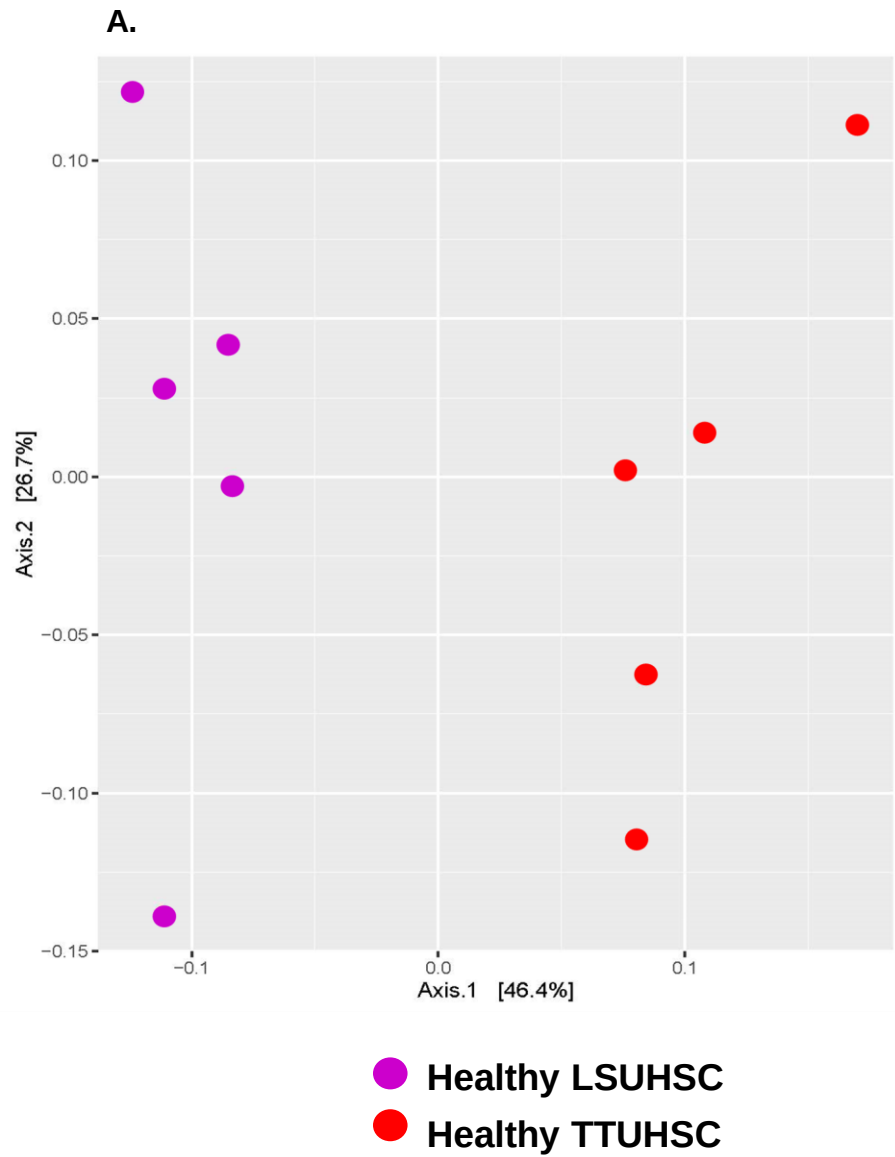


Figure 3

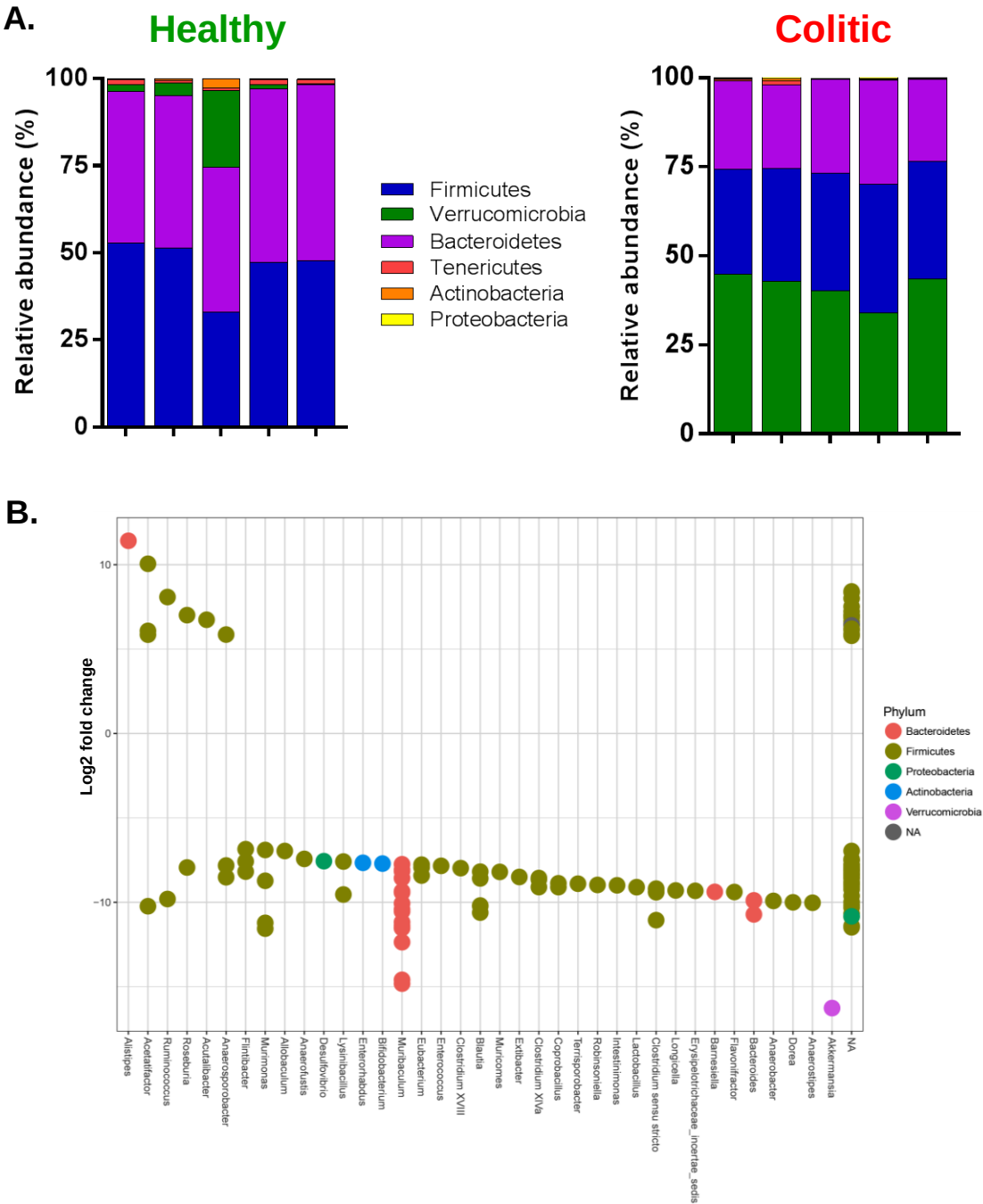
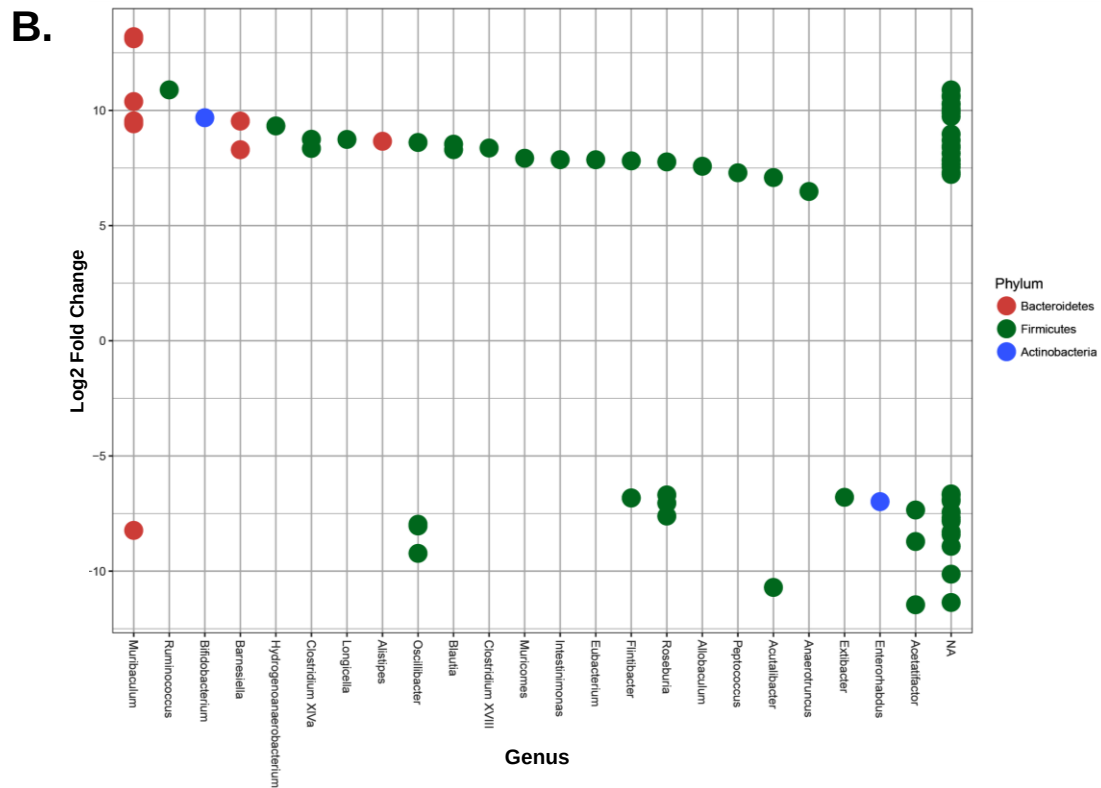
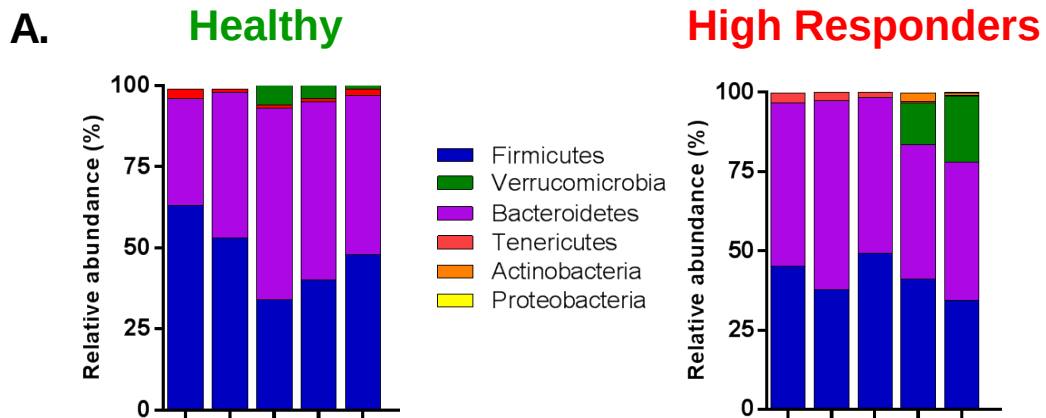


Figure 4



**A.**



A scatter plot showing the Log<sub>2</sub> Fold Change for various bacterial genera. The y-axis ranges from -10 to 10, and the x-axis lists the genera. Points are colored by phylum: Bacteroidetes (red), Tenericutes (brown), Firmicutes (green), Actinobacteria (teal), Proteobacteria (blue), and Verrucomicrobia (pink). Most genera show a negative fold change, indicating downregulation, while a few like Akkermansia and Clostridium XVIII show positive fold changes.

| Genus                     | Phylum        | Log <sub>2</sub> Fold Change (approx.) |
|---------------------------|---------------|----------------------------------------|
| Muribaculum               | Bacteroidetes | -16.5                                  |
| Anaeoplasma               | Bacteroidetes | -11.5                                  |
| Ruminococcus              | Firmicutes    | -9.8                                   |
| Syntrophococcus           | Firmicutes    | -9.2                                   |
| Barnesiella               | Bacteroidetes | -10.2                                  |
| Blautia                   | Firmicutes    | -9.5                                   |
| Flintbacter               | Firmicutes    | -8.8                                   |
| Anaeosporobacter          | Firmicutes    | -7.8                                   |
| Flavonifractor            | Firmicutes    | -7.5                                   |
| Oscillibacter             | Firmicutes    | -7.2                                   |
| Exibacter                 | Firmicutes    | -7.0                                   |
| Clostridium XIVa          | Firmicutes    | -6.8                                   |
| Enterococcus              | Firmicutes    | -6.5                                   |
| Coprocalillus             | Firmicutes    | -6.2                                   |
| Anaeostipes               | Firmicutes    | -6.0                                   |
| Eubacterium               | Firmicutes    | -5.8                                   |
| Lactobacillus             | Firmicutes    | -5.5                                   |
| Mutinus                   | Firmicutes    | -5.2                                   |
| Terrisporobacter          | Firmicutes    | -5.0                                   |
| Acetatifactor             | Firmicutes    | -4.8                                   |
| Robinsonella              | Firmicutes    | -4.5                                   |
| Clostridium sensu stricto | Firmicutes    | -4.2                                   |
| Longicella                | Firmicutes    | -4.0                                   |
| Dorea                     | Firmicutes    | -3.8                                   |
| Alisipies                 | Firmicutes    | -3.5                                   |
| Turbidbacter              | Firmicutes    | -3.2                                   |
| Clostridium XVIII         | Firmicutes    | -3.0                                   |
| Akkermansia               | Firmicutes    | 7.5                                    |
| NA                        | Firmicutes    | 8.0                                    |

Figure 6

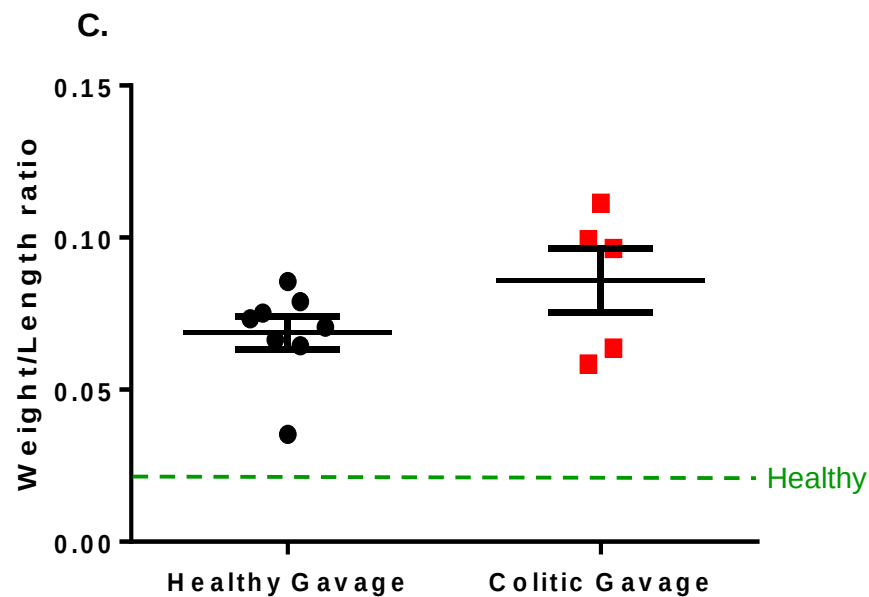
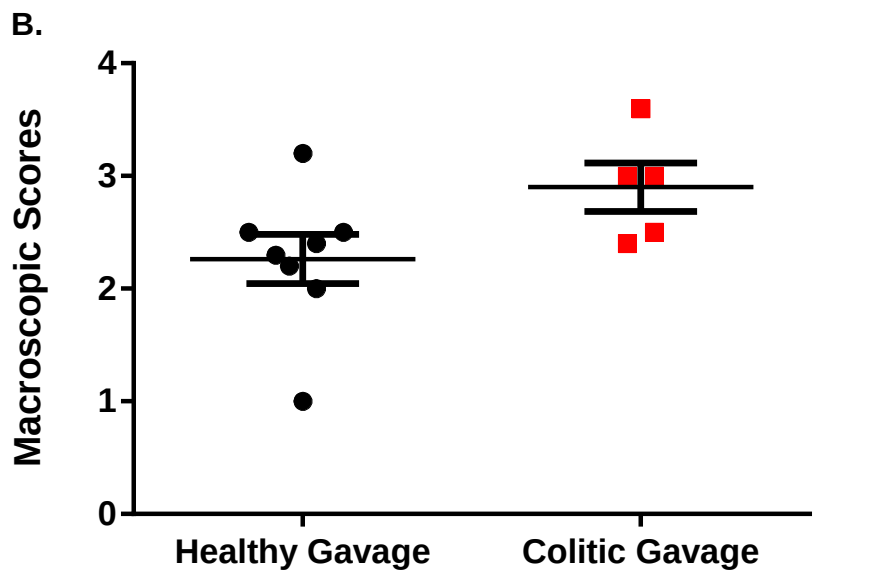
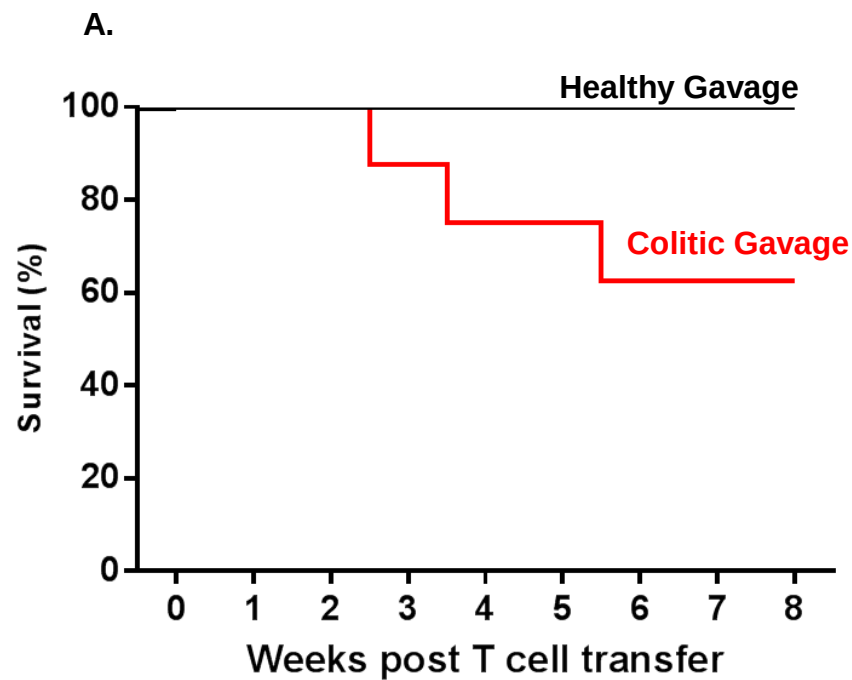
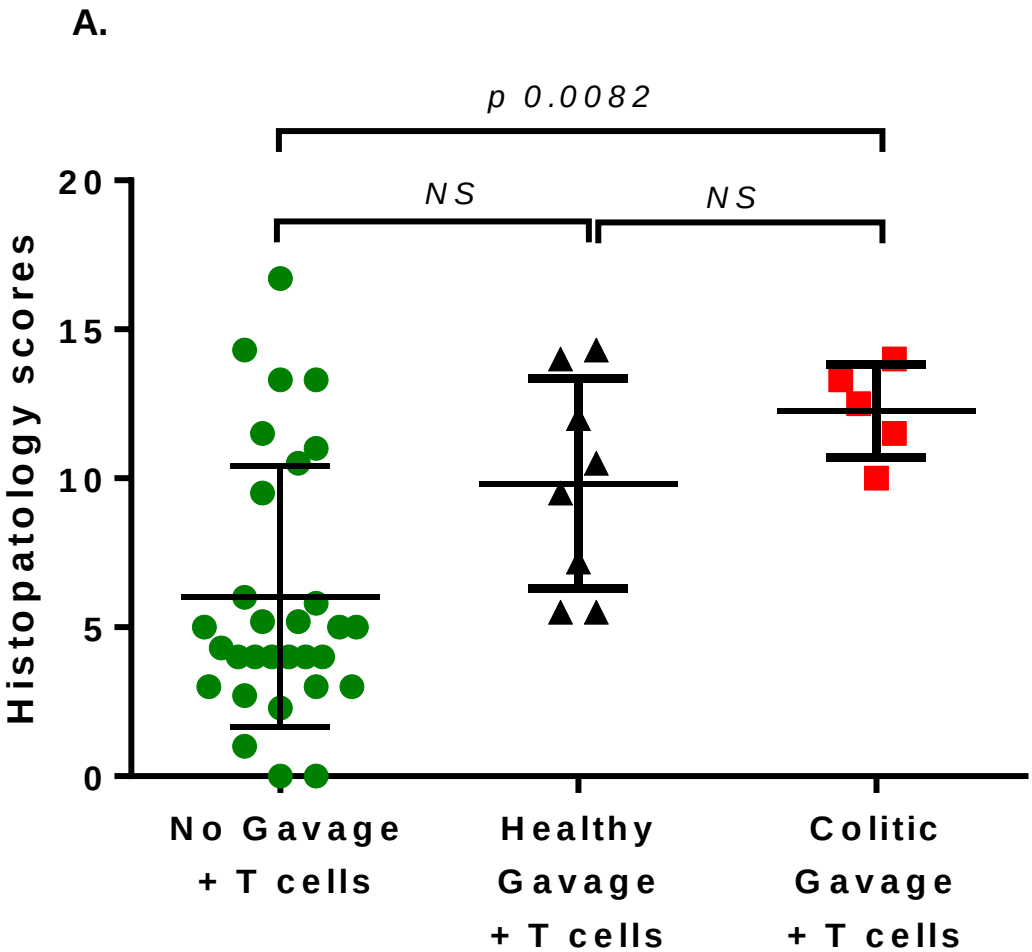
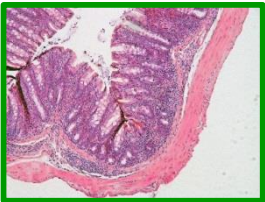


Figure 7

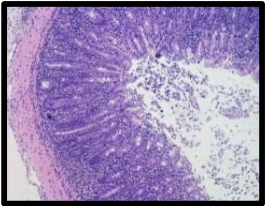


B.

No Gavage



Healthy Gavage



Colitic Gavage

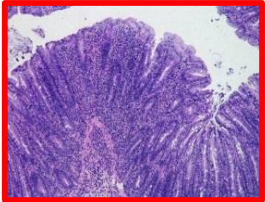


Figure 8

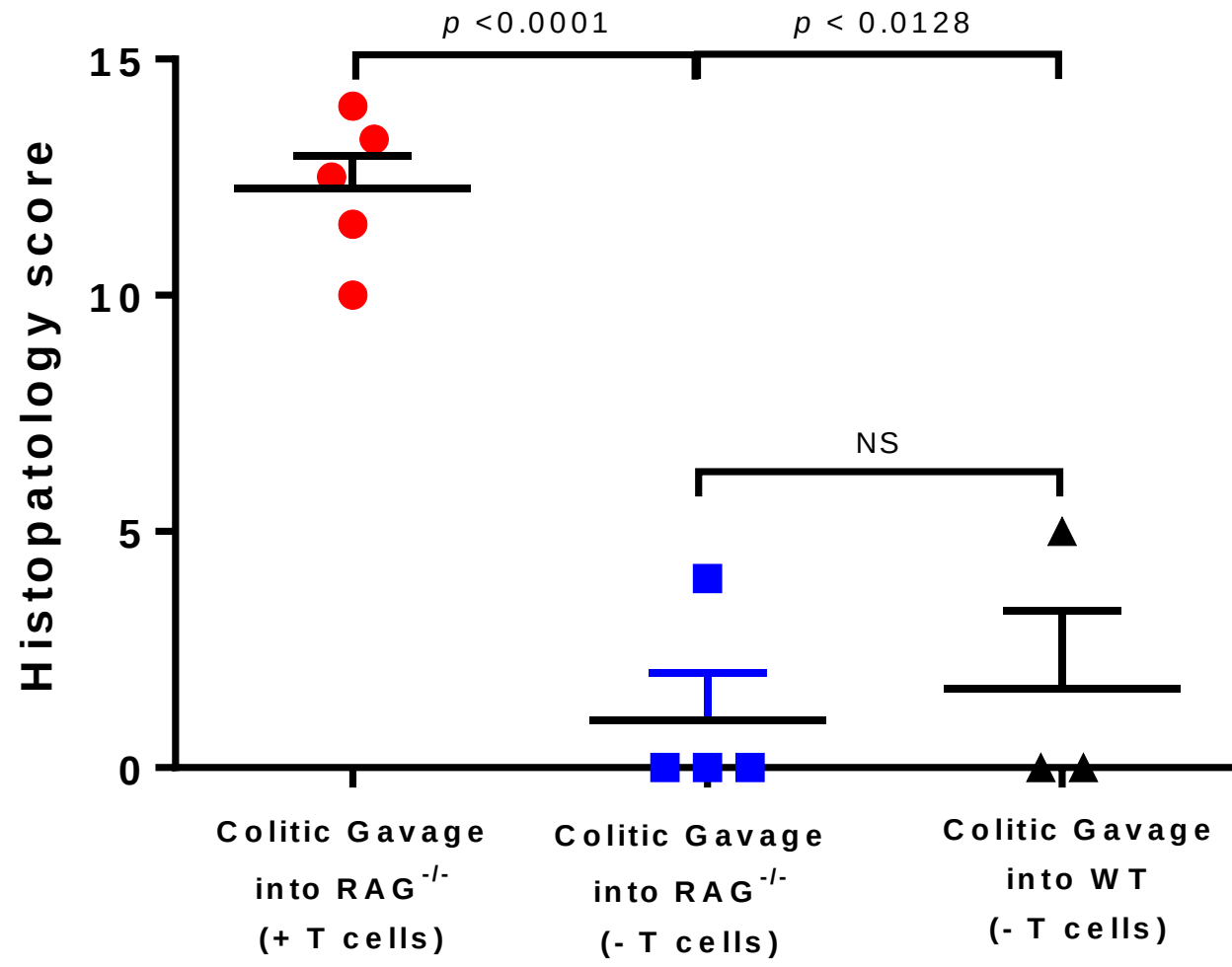
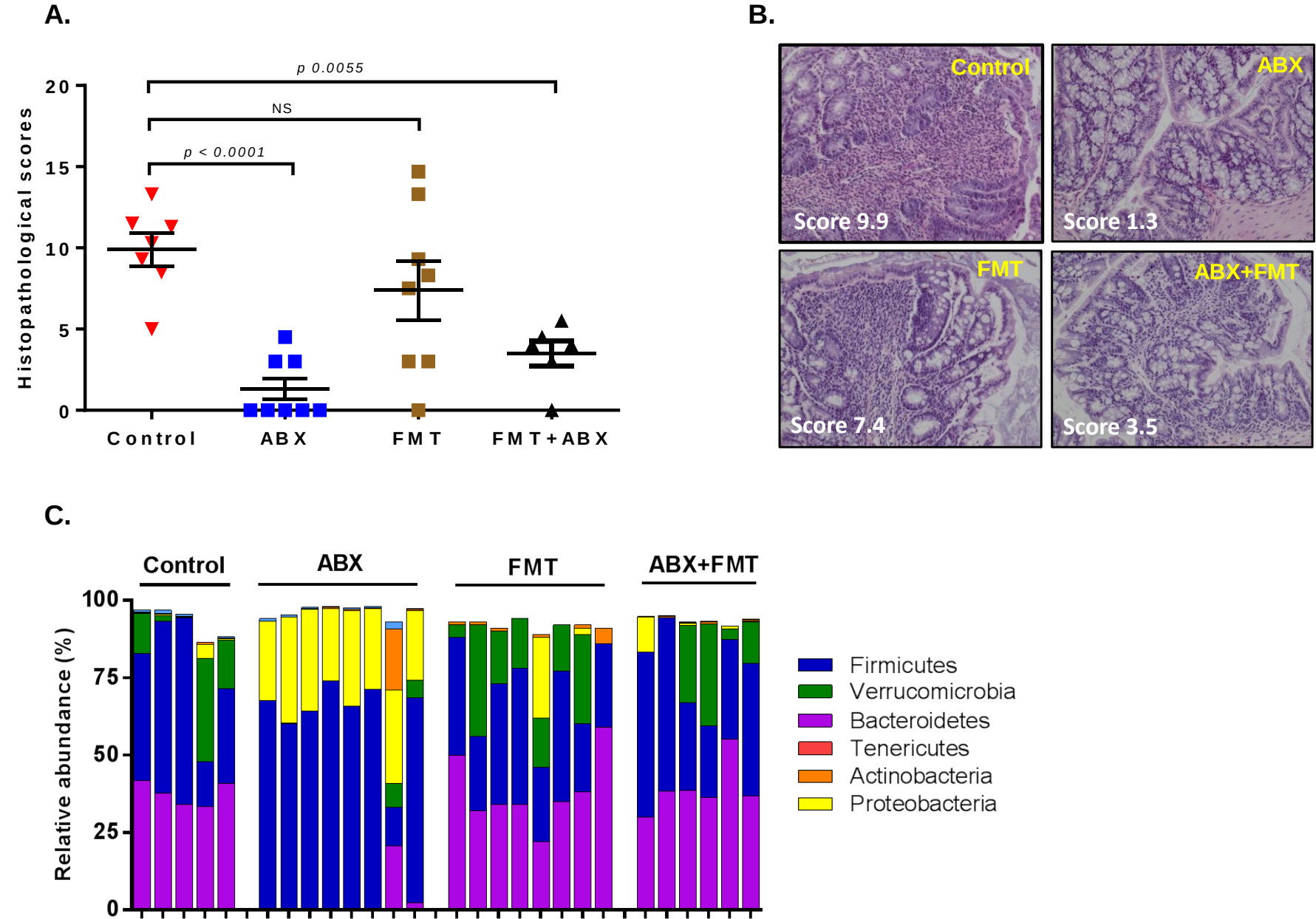
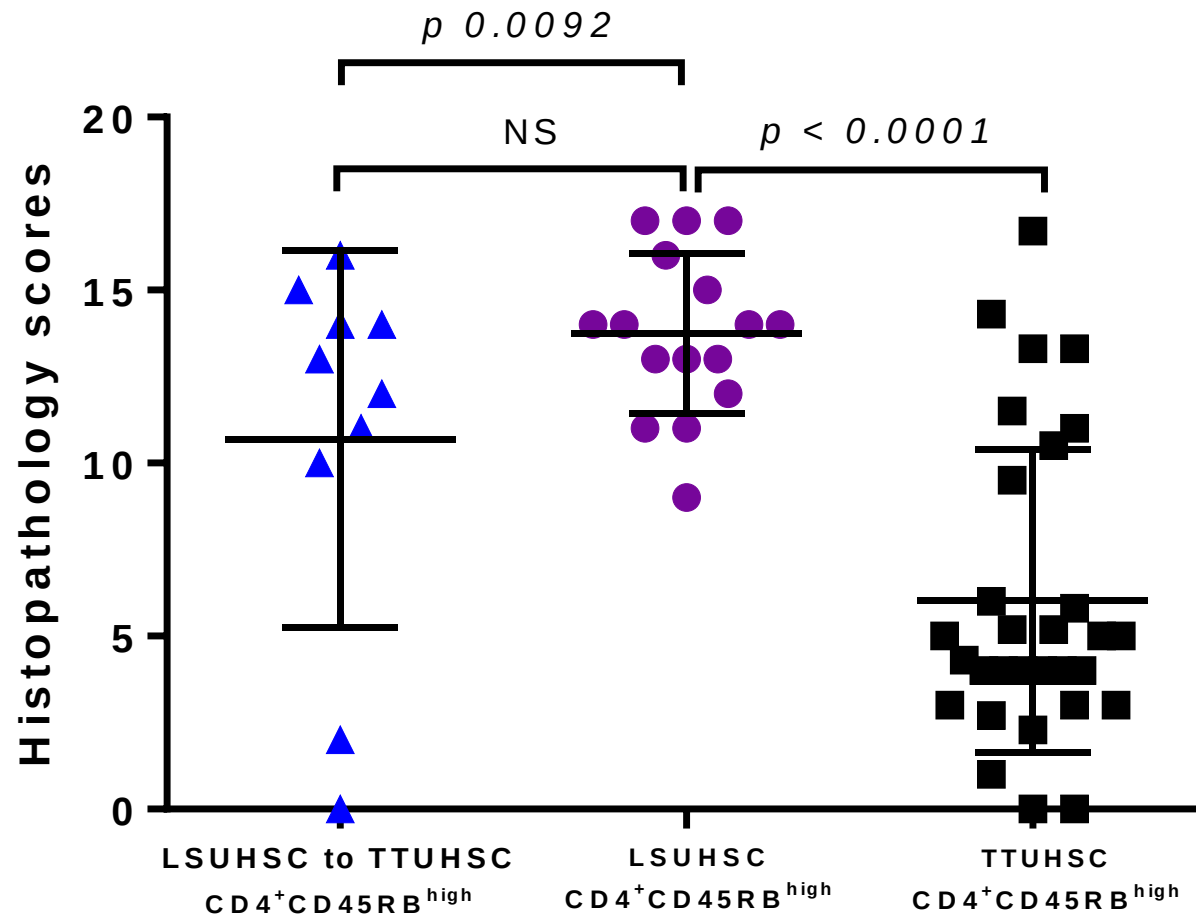
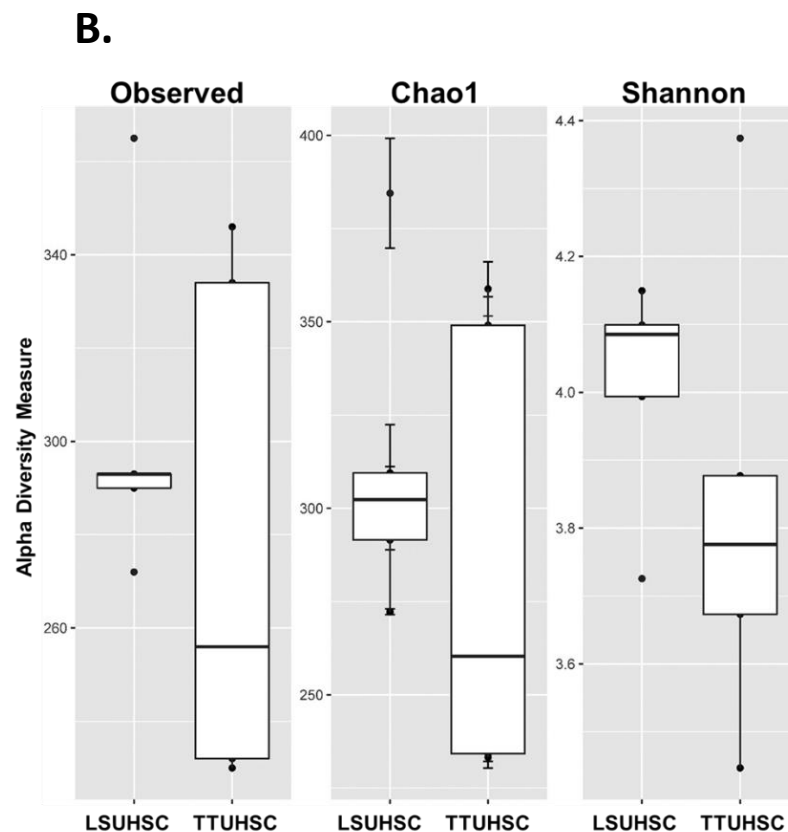
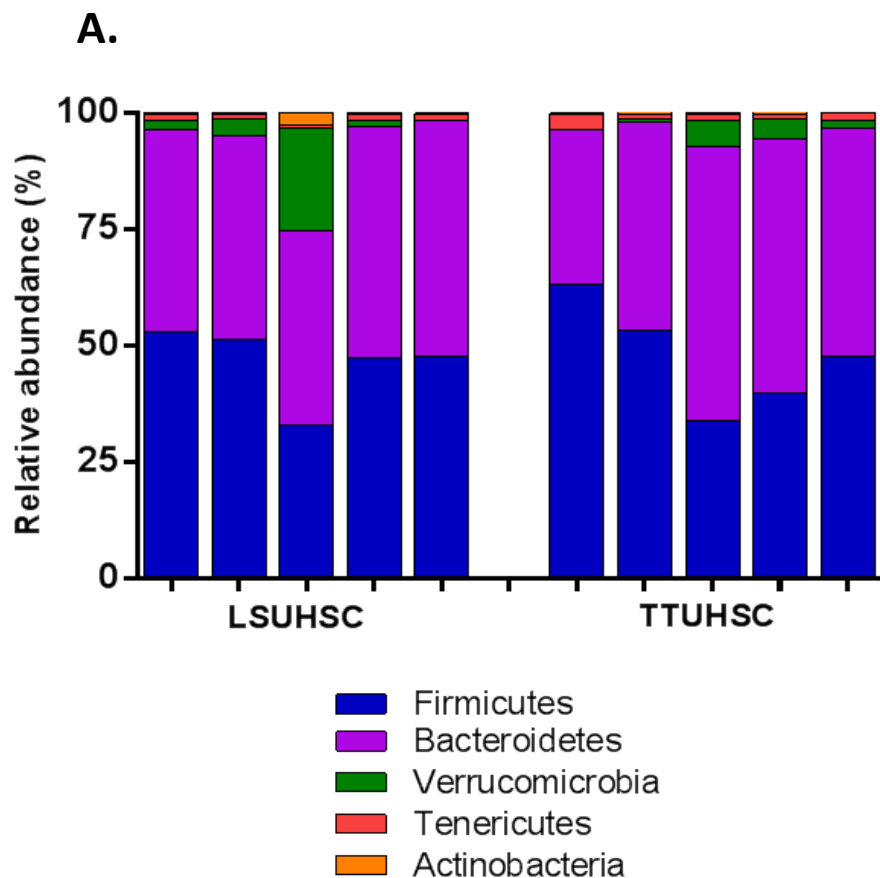


Figure 9



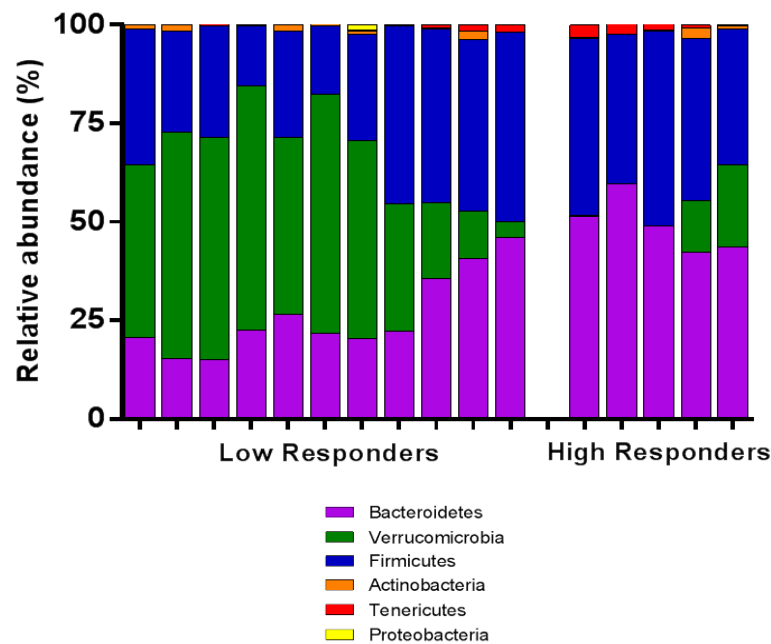


**Supplemental Figure 1. Chronic colitis develops in TTUHSC mice provided that T cell transfer occurs at LSUHSC two weeks prior to transfer.** Blinded histopathology scores of mice that engrafted with T cells at LSUHSC and then transferred to TTUHSC at two weeks following T cell engraftment. Data from Figure 1A are included for comparison. All histopathology scores were determined at 8 weeks post T cell transfer. Data are expressed as the mean  $\pm$  SEM. A total of 10, 16 and 30 mice were used for the LSUHSC to TTUHSC, LSUHSC and TTUHSC groups, respectively. Each experiment was repeated twice.

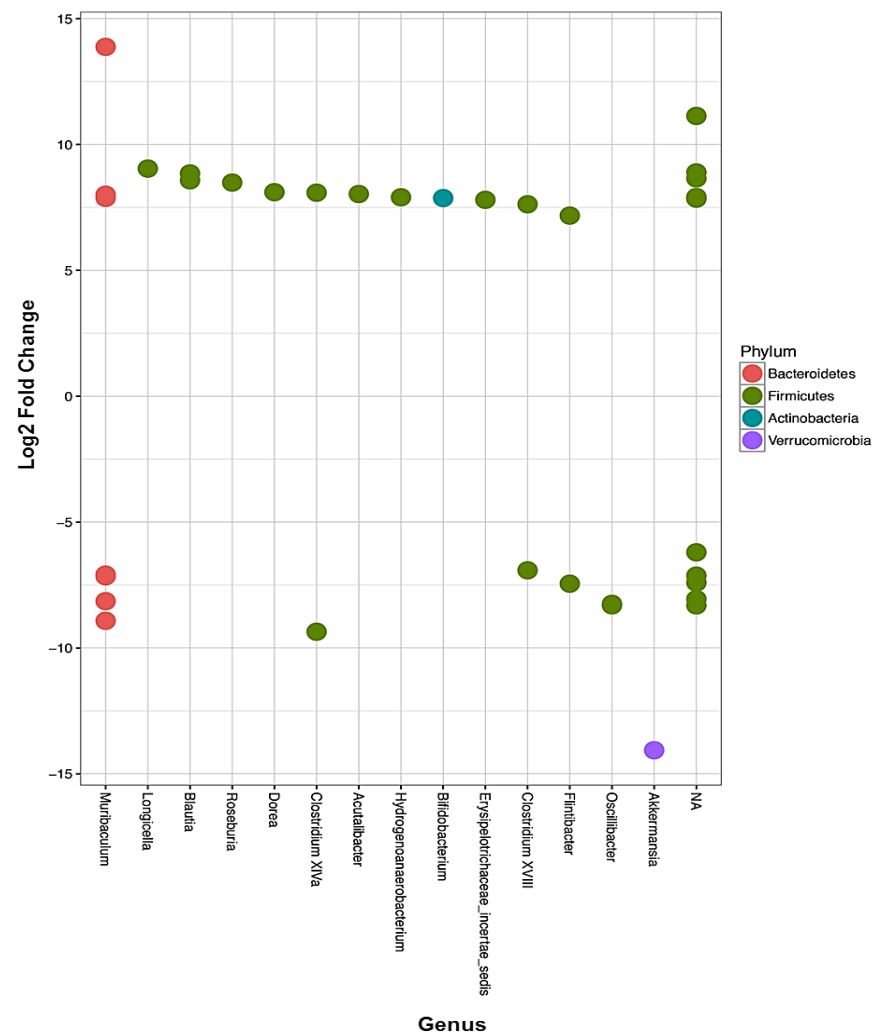


**Supplemental Figure 2. Taxonomic and alpha diversity analyses of fecal microbiota from healthy  $RAG^{-/-}$  mice housed at LSUHSC or TTUHSC. A).** Relative abundance of the 5 major phyla in feces obtained from  $RAG^{-/-}$  mice housed at LSUHSC or TTUHSC. **B).** Boxplots of OTU community richness and diversity as measured by Observed species, Chao1 and Shannon diversity indices (97% sequence similarity threshold). Analyses were performed using fecal pellets obtained from five different samples (LSUHSC) and from five different mice (TTUHSC).

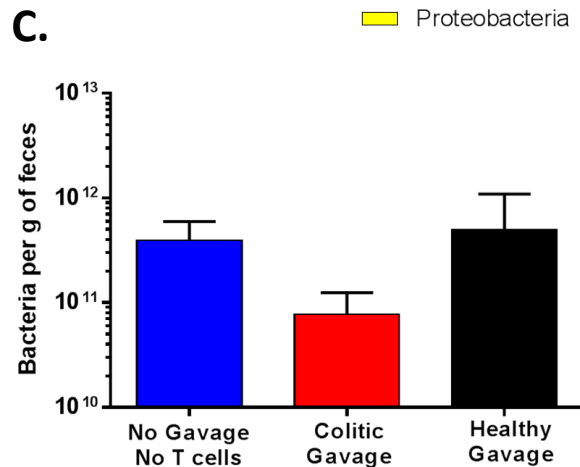
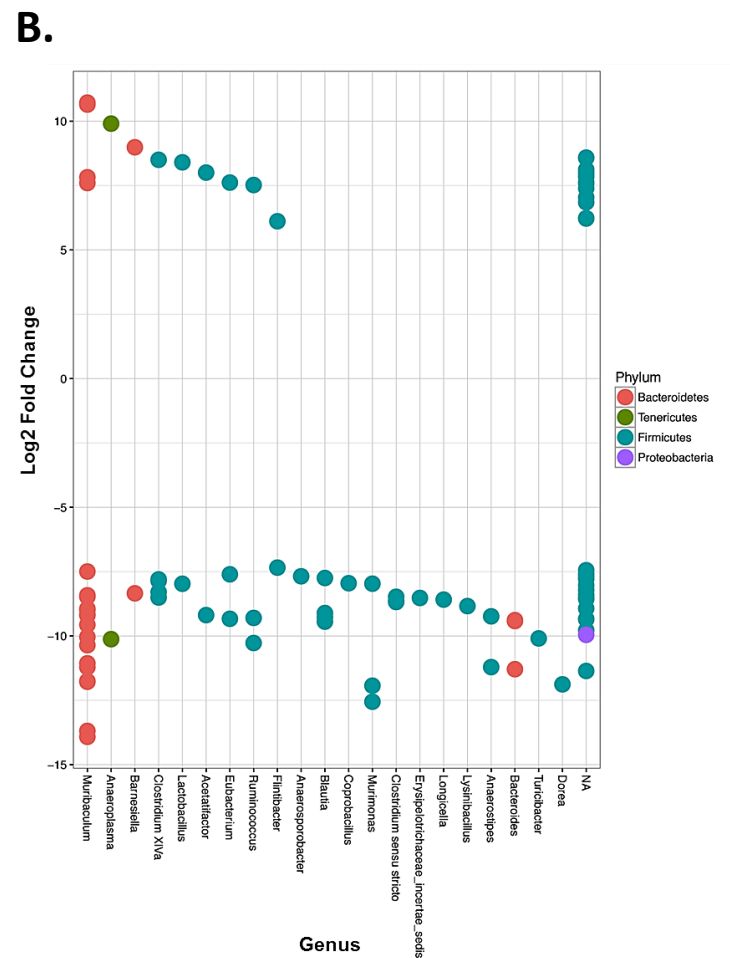
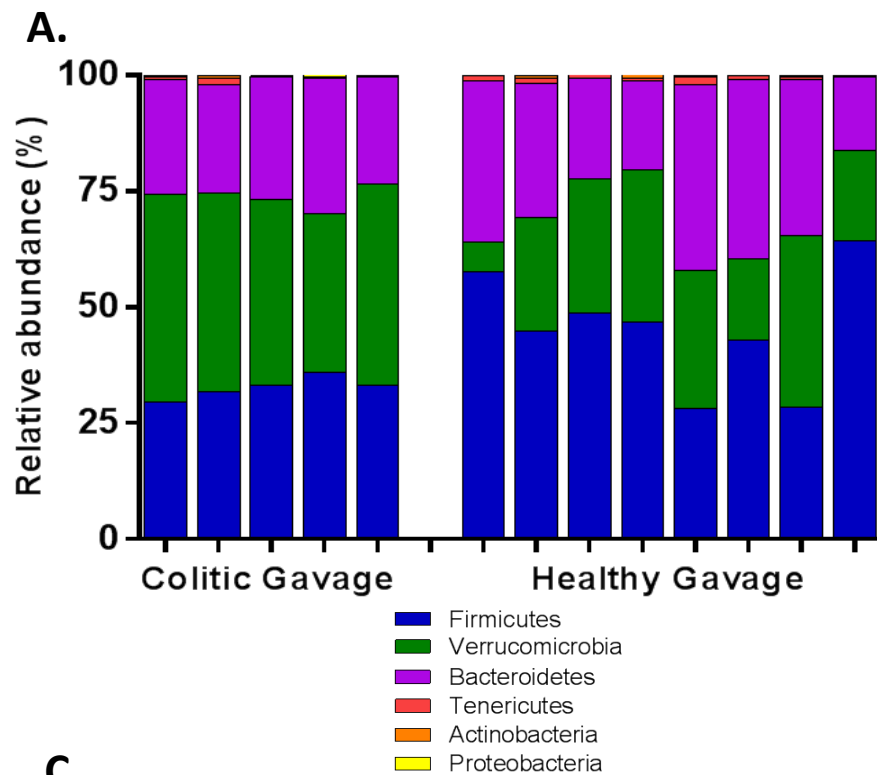
**A.**



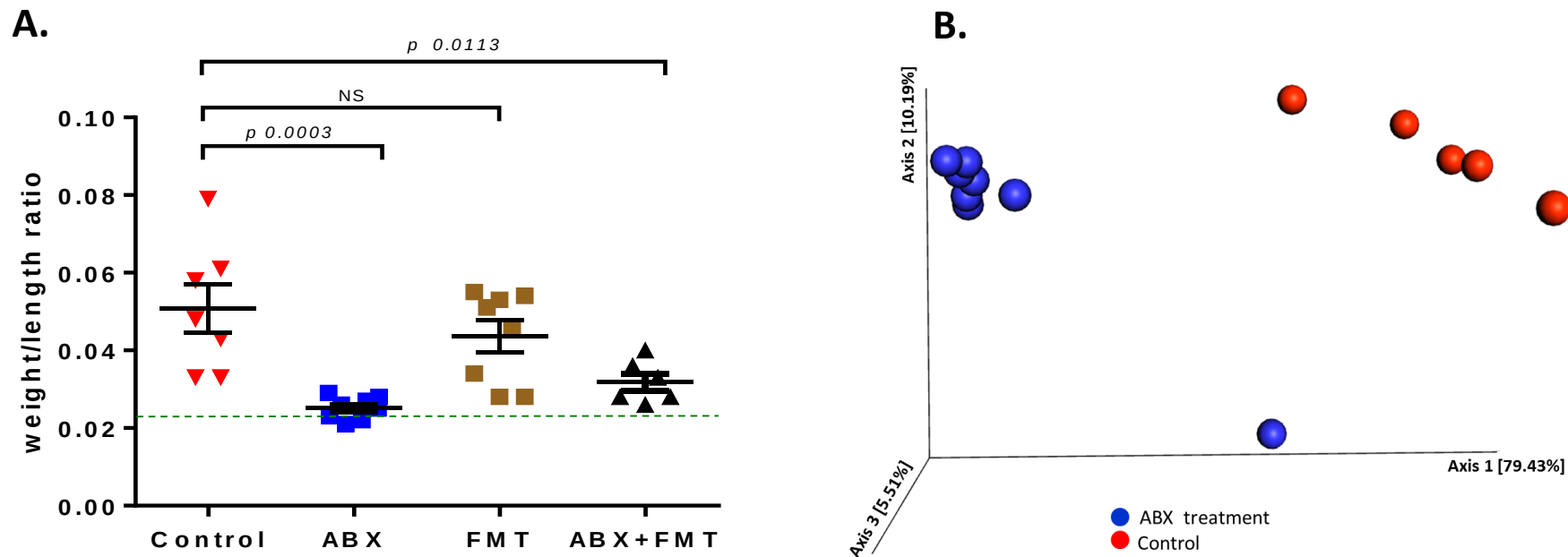
**B.**



**Supplemental Figure 3. Taxonomic and DESeq analyses of fecal microbiota from T cell-engrafted RAG<sup>-/-</sup> mice housed at TTUHSC. A).** Relative abundance of the six major phyla present in the low responder (histopathological scores <7; N=11 mice) and high responder (histopathological scores >7; N=5 mice) mice generated at TTUHSC. **B).** Differential overexpression of OTUs (genera) in feces obtained from TTUHSC high vs. low responders. Using DESeq analysis, values >0 represent OTUs (genera) that are significantly overexpressed in high responders, whereas values <0 represent genera that are significantly overexpressed in low responders. OTUs that exhibited significant (<0.01 multiple comparison adjusted p-value) within and between groups at the genus level between the two groups are shown. Multiple dots aligning within one genera represent different strains and/or species.



**Supplemental Figure 4. Fecal bacterial analyses in mice colonized with healthy or colitic feces obtained from mice housed at LSUHSC.** Recipient RAG<sup>-/-</sup> mice housed at TTUHSC were colonized (via gavage) with feces from healthy or colitic RAG<sup>-/-</sup> mice housed at LSUHSC prior to T cell transfer (see methods section). Analyses were performed at 8 weeks following T cell transfer. **A).** Relative abundance of the six major phyla present in feces from T cell-engrafted RAG<sup>-/-</sup> mice gavaged with healthy (N=8) or colitic (N=5) feces from LSUHSC mice. Each bar represents one animal. **B).** Differential overexpression of OTUs (genera) in feces obtained from T cell-engrafted mice that were gavaged with healthy or colitic feces. DESeq analysis ascribed values >0 to genera significantly enriched in mice gavaged with healthy feces whereas values <0 represented genera that were enriched in mice gavaged with colitic feces. **C)** Fecal bacterial load in T cell-engrafted mice colonized (via gavage) with healthy (N=8) or colitic (N=5) feces.



**Supplemental Figure 5. Therapeutic effects of antibiotic treatment and/or fecal microbiota transplant on colonic weight/length ratios and microbial beta diversity.** Treatment with antibiotics (ABX and vancomycin; N=8), fecal microbial transplant (FMT; N=9) or both ABX and FMT (N=5) began 4 weeks following T cell transfer as described in the Methods section. Seven mice were not treated and served as the untreated control group. **A).** Colon weight/length ratios were quantified and used as an indicator of colonic inflammation. Data are expressed as the mean  $\pm$  SEM. Healthy mouse colons exhibit a colon weight/length ratio of  $\sim 0.20$  (indicated with dashed green line). **B).** Principal coordinate analysis (PCoA) based on weighted UniFrac for antibiotic-treated (ABX) vs. untreated (controls) mice. Notice the clear separation of the ABX treated mice from the control group with the exception of one outlier.

## Supplemental Table S1

### Comparison of Rodent Chows used at LSUHSC and TTUHSC

|                                 | <b>Teklab (Envigo)<br/>7012 LM-485<br/>(LSUHSC)*</b> | <b>Prolab<br/>RMH 3000-5P75<br/>(TTUHSC)#</b> |
|---------------------------------|------------------------------------------------------|-----------------------------------------------|
| <b>Ingredients</b>              | Ground corn                                          | Soybean meal                                  |
|                                 | Soybean meal                                         | Ground corn                                   |
|                                 | Ground oats                                          | Wheat middlings                               |
|                                 | Wheat middlings                                      | Porcine animal fat (preserved<br>with BHA)    |
|                                 | Alfalfa meal                                         | Fish meal                                     |
|                                 | Corn gluten meal                                     |                                               |
|                                 | Brewers yeasts                                       |                                               |
|                                 |                                                      |                                               |
| <b>Isoflavone content</b>       | 300-600 mg/kg                                        | not indicated                                 |
|                                 |                                                      |                                               |
| <b>Protein</b>                  | 19.1%                                                | 26.0%                                         |
| <b>Fat</b>                      | 5.8%                                                 | 14.3%                                         |
| <b>Carbohydrate</b>             | 44.3%                                                | 59.7%                                         |
| <b>Source of fat</b>            | Soybean oil                                          | Soybean oil                                   |
|                                 |                                                      |                                               |
| <b>Metabolizable<br/>energy</b> | 3.1 kcal/g                                           | 3.2 kcal/g                                    |

\*Obtained from Teklab (Envigo) Website

#Obtained from Prolab Website

## Appendix Item 2

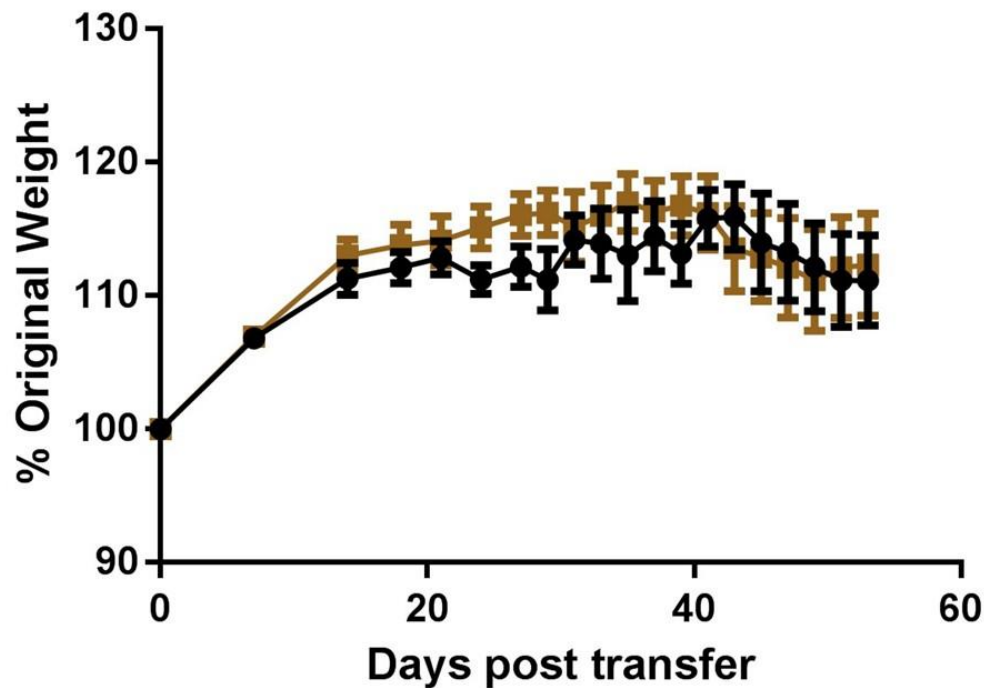


Figure 1. Changes in body weight of mice treated with either PBS (brown squares) or human MSCs (black circles). RAG<sup>-/-</sup> mice were reconstituted with naïve T cells to induce chronic colitis and then injected (i.v.) mice with PBS or MSCs ( $1.3 \times 10^6$  cell) 4 different times over a 2 week period beginning 2 weeks post T cell transfer.

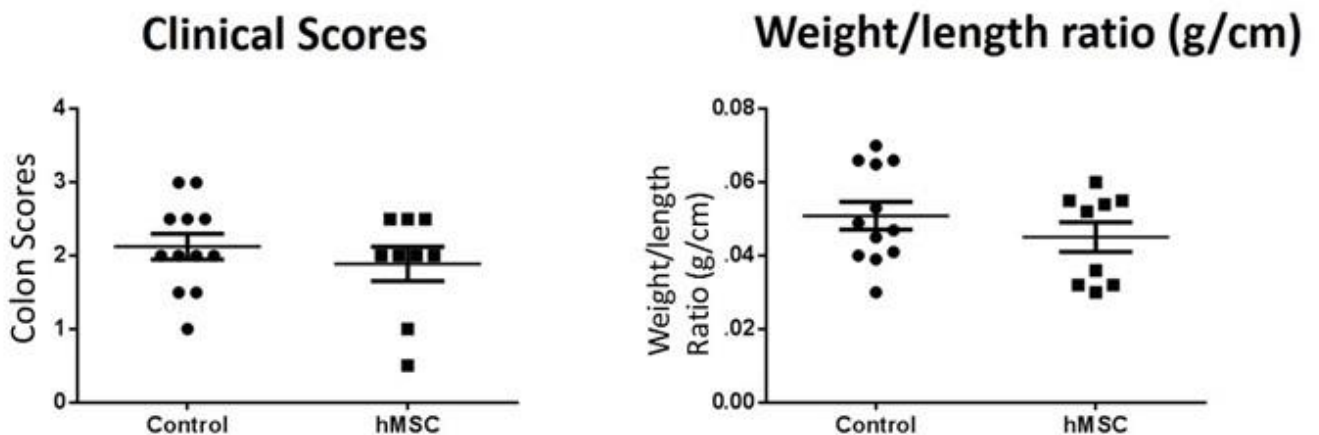


Figure 2. Clinical scores and weight-to-length ratios of colons from mice treated with either PBS (control) or human MSCs (hMSCs). Chronic colitis was induced in RAG<sup>-/-</sup> mice and PBS or MSC treated was performed as described in Figure 1. Colons were obtained from mice euthanized at 8 weeks following T cell transfer.

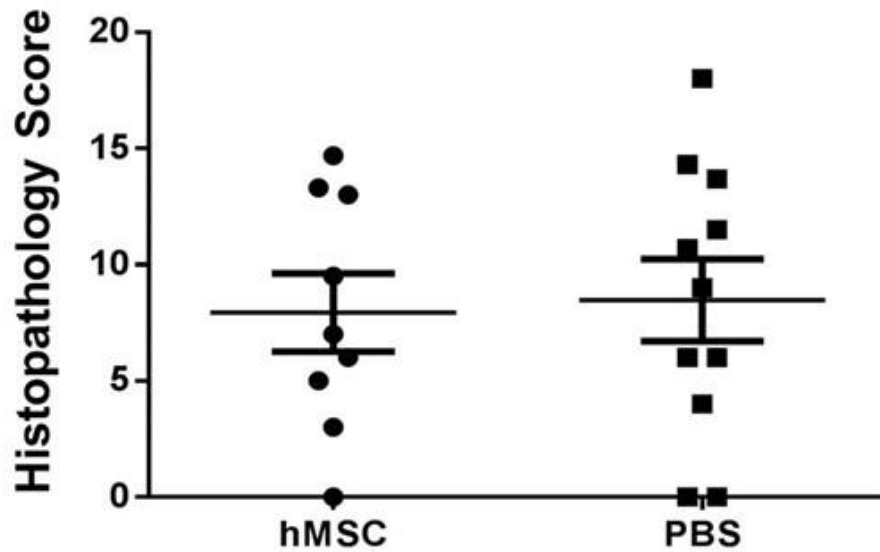


Figure 3. Blinded histopathology scores of colons from mice treated with either human MSCs (hMSCs) for PBS. Chronic colitis was induced in RAG<sup>-/-</sup> mice and PBS or MSC treatment was performed as described in Figure 1. Colons were obtained from mice euthanized at 8 weeks following T cell transfer.

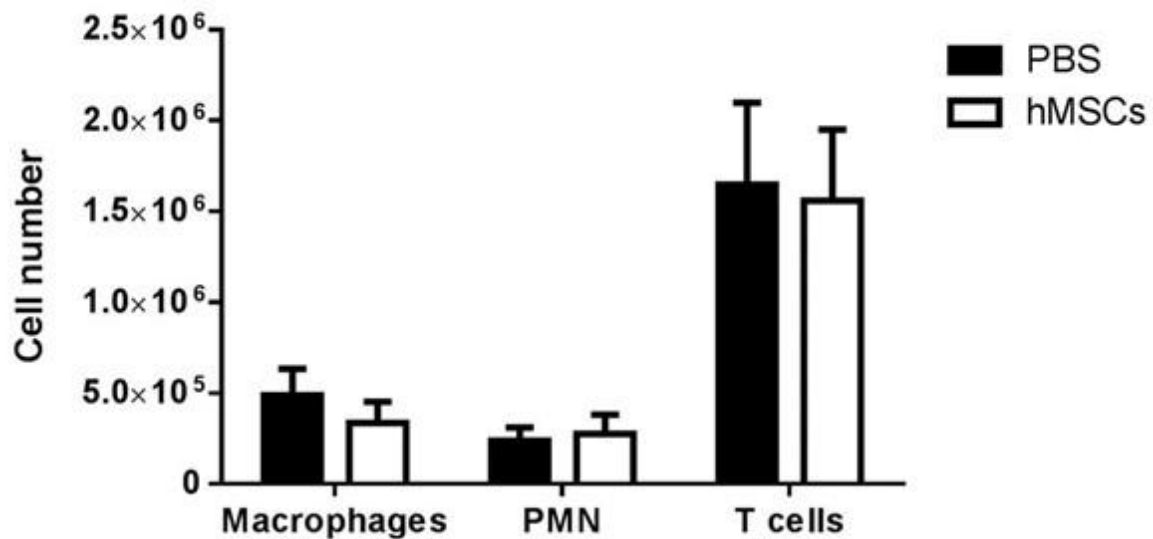


Figure 4. Leukocyte numbers in colonic lamina propria of mice treated with either PBS or human MSCs (hMSCs). Chronic colitis was induced in RAG<sup>-/-</sup> mice and PBS or MSC treatment was performed as described in Figure 1. Colons were obtained from mice euthanized at 8 weeks following T cell transfer.

# **Appendix Items 3-5**

## **Publications**



## Review

## Protective and pro-inflammatory roles of intestinal bacteria

Cynthia Reinoso Webb<sup>1</sup>, Iurii Koboziev<sup>1</sup>, Kathryn L. Furr, Matthew B. Grisham<sup>\*</sup>

Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, United States

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## ABSTRACT

The intestinal mucosal surface in all vertebrates is exposed to enormous numbers of microorganisms that include bacteria, archaea, fungi and viruses. Coexistence of the host with the gut microbiota represents an active and mutually beneficial relationship that helps to shape the mucosal and systemic immune systems of both mammals and teleosts (ray-finned fish). Due to the potential for enteric microorganisms to invade intestinal tissue and induce local and/or systemic inflammation, the mucosal immune system has developed a number of protective mechanisms that allow the host to mount an appropriate immune response to invading bacteria, while limiting bystander tissue injury associated with these immune responses. Failure to properly regulate mucosal immunity is thought to be responsible for the development of chronic intestinal inflammation. The objective of this review is to present our current understanding of the role that intestinal bacteria play in vertebrate health and disease. While our primary focus will be humans and mice, we also present the new and exciting comparative studies being performed in zebrafish to model host–microbe interactions.

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## 1. Introduction

The healthy human body contains 10 times more microbial cells than human cells! This proclamation has been repeated many times over the past several years in both the scientific literature, as well as the lay press [169]. Although this declaration has been presented as a scientific fact over the past decade, it may not be

entirely accurate in view of a recent reexamination of the published data [169]. Most reviews that focus on host–microbe interactions begin with the statement that the healthy human intestine contains approximately 100 trillion ( $10^{14}$ ) microbes [55,102,176,178,211]. Furthermore, many of these publications state, without reference, that the total number of human cells in the body approximates 10 trillion ( $10^{13}$ ) cells [169,178]. However, this 10-fold excess of microbial to human cells may need to be reevaluated based upon more recent work that has been largely overlooked during the past few years. For example, the statement that the intestinal tract contains  $10^{14}$  microbial cells is based upon a 44 year-old report that provides little by way of direct quantitative data for this fecal

<sup>\*</sup> Corresponding author at: Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, 3601 4th Street STOP 6591, Lubbock, TX 79430, United States.

E-mail address: [matthew.grisham@ttuhsc.edu](mailto:matthew.grisham@ttuhsc.edu) (M.B. Grisham).

<sup>1</sup> These authors contributed equally to the preparation of this manuscript.

bacterial estimate [108]. Using more sophisticated technology, Suau et al. have determined that the numbers of bacteria that reside within the healthy human intestinal tract range from  $3 \times 10^{13}$  to  $40 \times 10^{13}$  (30–400 trillion) [194]. The assertion that humans contain  $10^{13}$  body cells is based upon one sentence from a 46 year-old book that provides no experimental data nor references for this estimate [37]. A recent study by Bianconi et al. using systematic quantification of cell numbers in different tissues reports that humans contain, on average, 30–40 trillion body cells [12]. These newer data would suggest a more realistic ratio of microbial to human cells that range from 1:1 to 10:1.

While these more quantitative estimates are, in some cases, quite different from what has been repeatedly stated in scientific and lay publications, they confirm that the gut is home to enormous numbers of bacteria. The large majority (>90%) of intestinal bacteria in the human intestinal tract belongs to the phyla Bacteroidetes and Firmicutes. However, substantial numbers of bacteria belonging to phyla Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia and Cyanobacteria are also observed [38,40,44,102,106,176] (Fig. 1). In addition to the enormous population of bacteria, the human gut has also been estimated to contain more than a quadrillion ( $10^{15}$ ) viruses and bacteriophages, as well as substantial numbers of archaea and fungi [40,55,62,102,106,143,199]. Most of the detailed characterization and bioinformatic analyses of the intestinal microbiota have been performed using human stool and/or mucosal tissue. However, mice have also been extensively used to define the importance of host genetics, microbiota and the immune system in homeostasis and disease [28,141]. The use of mice provide investigators with a small animal model to assess, in a well-controlled environment, the complex host–microbe interactions that occur *in vivo*. While mice and humans share two major phyla (Bacteroidetes and Firmicutes) and approximately 80 different genera [141,168], major differences exist among bacterial species in these two mammals [141,168].

Valuable information has been generated using gnotobiotic and fully colonized mice to assess host–microbiome interactions. However, these studies are limited by the length of time and the high cost associated with the generation of large numbers of genetically-manipulated animals required to yield statistically-powered *in vivo* studies. In an attempt to shorten the time and cost of new discoveries, investigators have begun to use other vertebrates to model these interactions in healthy and inflamed intestine. For example, zebrafish (*Danio rerio*) have become increasingly popular for these types of studies given the similarity of their intestinal tract to that of mammals [57,208]. Although major differences exist between teleost and mammal microbiota, zebrafish share many of the major microbial communities that have been identified in rodents and humans [160,161,180,195,198]. Investigators have shown that similar to humans and mice, the teleost gut contains large numbers of Proteobacteria, Firmicutes and Bacteroidetes [30,166,195] (Fig. 1). The use of teleosts offer a number of advantages over mice and other rodents due to the relatively low cost to produce and maintain large numbers of larvae and adults, their accelerated development, and their transparent skin that allows for detailed and noninvasive imaging studies [161,216]. Another major advantage of zebrafish is their amenability to produce forward and reverse genetic manipulations [216]. Furthermore, because these vertebrates live in an aqueous environment, the delivery of different chemicals/small molecules, therapeutic agents or microbiota to germ-free or fully colonized zebrafish is relatively a straight forward process [19,47,64,160,161,198].

The continuous exposure of the vertebrate intestine to such large and diverse populations of microorganisms in close proximity to a tissue that contains large numbers of immune cells, makes the gut the largest and most complex component of the immune system. The coexistence of vertebrates with their gut microbiota

is a dynamic and mutually beneficial relationship that plays an important role in the well-being of the host [29]. However, the close proximity of potentially harmful/pathogenic microorganisms has forced the intestinal immune system to develop a number of different immune mechanisms to eliminate invading microbes, while suppressing the bystander tissue injury associated with these innate and adaptive immune responses. Failure to properly regulate these protective immune responses may induce chronic inflammatory responses that are thought to be critical immunopathological mechanisms responsible for the development of human inflammatory bowel diseases (IBD; Crohn's disease, ulcerative colitis). These idiopathic inflammatory diseases affect primarily the small and/or large bowel and are characterized by the infiltration of large numbers of inflammatory leukocytes (e.g., neutrophils, monocytes, and lymphocytes) into the intestinal lamina propria (LP) where they directly or indirectly promote inflammation with tissue injury, loss of goblet cells, fibrosis, erosions and ulcerations. Although the etiology of IBD remains to be defined, it is becoming increasingly appreciated that chronic intestinal inflammation may result from a complex interaction among genetic, immune and microbial factors [73,99,214]. Based upon a large body of experimental and clinical evidence generated over the past 20 years, investigators hypothesize that chronic gut inflammation results from a dysregulated immune response to components of the normal gut flora in genetically-susceptible individuals [31,79,95]. Although mouse models of IBD have been used for more than 20 years and have been instrumental in defining many of the major immunopathological mechanisms responsible for inflammatory tissue injury in these models, progress as been slow for reasons outlined above [95]. Thus, several groups of investigators have turned to the use of zebrafish to model IBD (see below) [19,47,63,64,216]. The objective of this review is to present our current understanding of the role that the intestinal microbiota plays in vertebrate intestinal health and inflammation. While our primary focus will be humans and mice, we also present the new and exciting comparative studies being performed in zebrafish to model host–microbe interactions.

## 2. Development of bacterial communities within the intestinal tract

The colonization and development of the intestinal microbiota in all vertebrates is crucial for the generation of a fully functional immune system, production of essential nutrients and vitamins, and metabolism of xenobiotics. While it has been assumed that the development of a stable microbiota in the mammalian gut begins at birth, since *in utero* the fetus has been thought to be germ-free, more recent reports suggest that this may not be the case as bacteria have been isolated from meconium, umbilical cord and amniotic fluid obtained from healthy pregnancies [81]. Nevertheless, the development of a newborn's microbiota begins following birth *via* the colonization of the infant's intestinal tract with bacteria associated with the mother's skin, vagina, feces, and breast milk [102,117,190]. During the first three months of life, *Bifidobacterium* and *Lactobacillus* colonize the intestinal tract in mammals due to the ingestion of breast milk [97,190]. Early on in the infant's life, the microbial communities are highly variable and relatively unstable when compared to the adult microbiota which has much greater complexity and phylogenetic diversity [97,149]. It is thought that the stabilization/maturation of the microbiota occurs at approximately 2–3 years of age and that the microbiota can be prepared with genes for the metabolism of food that is not yet being consumed by the infant (*i.e.*, plant polysaccharide metabolism) [6,97,149,217]. Koenig et al., has shown that the assembly of the microbial communities early in life is not random, but instead, occurs by way of specific bacterial successions due to different life

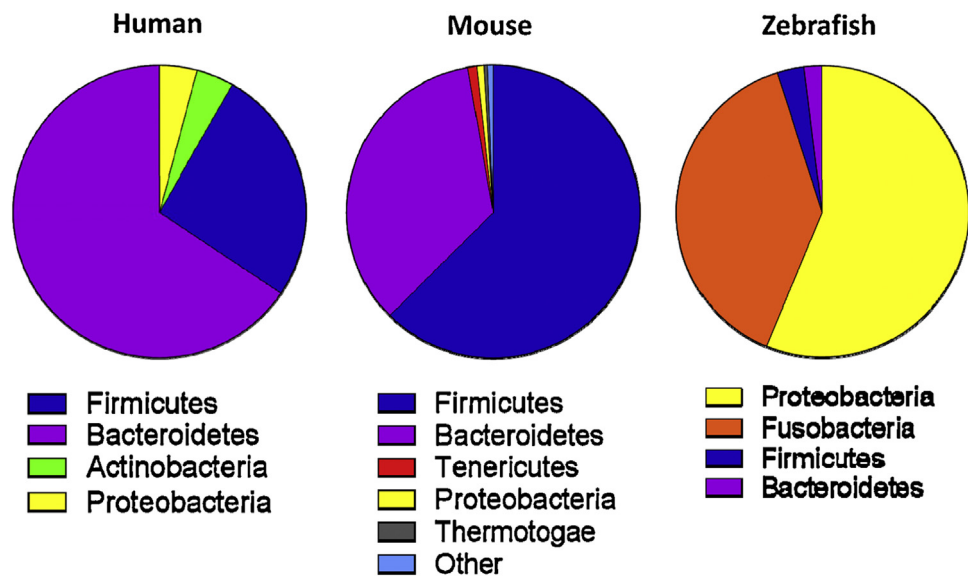


**Fig. 1.** Relative abundance of the major intestinal phyla in humans, mice and zebrafish. Deep sequencing of the 16S rRNA gene reveals that while the relative abundance of specific bacterial phyla may differ, mammals and teleosts possess similar bacterial communities. Human and zebrafish pie charts were derived from Refs. [121,166], respectively (with permission). Mouse pie chart was generated from colonic luminal contents of healthy C57Bl/6 mice housed at the TTUHSC animal facility (unpublished data).

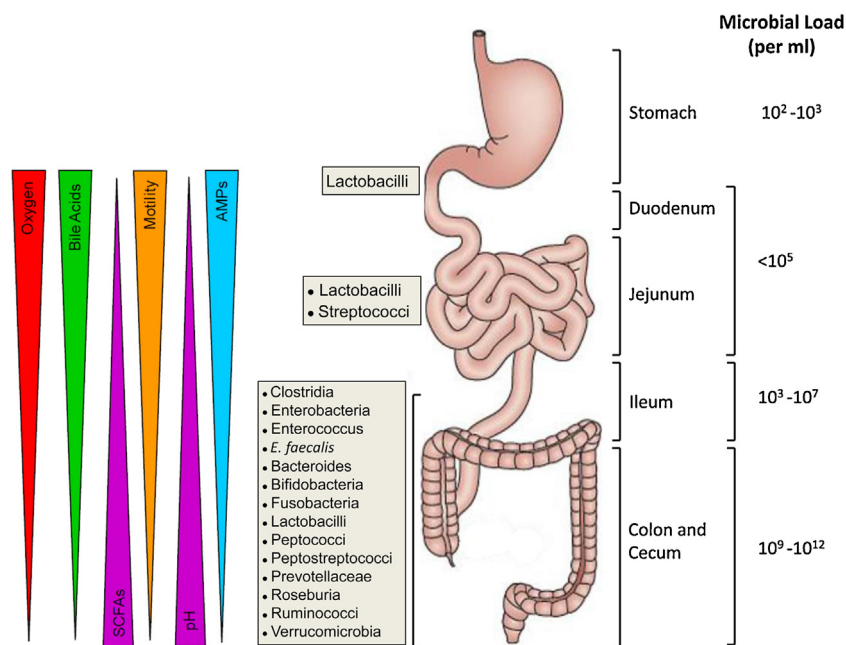


**Fig. 2.** Spatial organization of microbial communities and physiological gradients along the mammalian gastrointestinal tract. The numbers and types of bacterial communities, as well as physiological factors vary along the length of the gastrointestinal (GI) tract. It is well-appreciated that the oxygen levels, bile acid concentrations, intestinal motility, antimicrobial peptides (AMPs) and luminal pH in proximal portion of the GI tract (stomach, duodenum, jejunum) play major roles in restricting the numbers and types of microorganisms. In general, aerobic and facultative anaerobic bacteria are found almost exclusively in the proximal portion of the GI tract. The hypoxic nature and more physiological pH of the distal small intestine (ileum) and colon coupled to overall reductions of bile acids, AMPs and gut motility, allows for unfettered growth of large numbers of obligate anaerobic bacteria. These oxygen-sensitive microbes are capable of producing large quantities of short chain fatty acids (SCFAs; acetate, propionate, butyrate) from complex carbohydrates (fiber) to be used for important colonic and immunologic processes. Figure derived from Refs. [38,139,214]. The anatomical structure of the gastrointestinal tract with associated microbial communities and bacterial load were reproduced from Ref. [139] with permission.

events [97]. The fully mature intestinal microbiota is distinctly distributed among the different parts of the gastrointestinal (GI) tract, dictated primarily by the different oxygen concentrations found within the lumen and along the length of the GI tract (Fig. 2). It is well-appreciated that the more proximal portions of the GI tract (i.e., stomach, duodenum) contain much higher levels of luminal oxygen and are populated by a higher percentage of aerobic and facultative anaerobic bacteria; although total numbers of bacteria

in these regions of the GI tract are much lower than those found in the distal portions of the small and large bowel [42,176]. The distal portion of the small intestine as well as the colon contains little or no oxygen, thereby facilitating the growth of obligate anaerobes [1] (Fig. 2).

The early development of intestinal microbial communities in mammals contrasts that of teleosts, given their differences with respect to life cycle. For example, zebrafish egg fertilization takes

place externally with embryonic development occurring within the sterile chorion. At 2 days post fertilization (d.p.f.) the larvae hatch and are released into the environment where microbial colonization begins [33,219]. As with other vertebrates, microbial colonization of the intestine serves as a potent stimulus to induce the maturation of the gut immune system and digestive functions such that the larvae may begin to ingest food at approximately 5 d.p.f. [8,219]. The development of the intestinal microbiota continues throughout life, continuously responding to changes in the environment as the zebrafish mature from larva to adult. Although little information is currently available describing the intestinal microenvironment, its luminal oxygen tension, the regional microbial composition and total bacterial load in teleosts, it has been reported that the distribution of microbial communities in the hindgut of fish is similar to the distal bowel of mammals as they share similar communities of bacteria such as Proteobacteria, Fusobacteria, Firmicutes and Bacteroidetes [30,166,187,195]. Once established, intestinal microbial communities in both mammals and fish may be continually modified by different factors that include diet, genetics, sex and the environment [36,106,120,195]. Some of these factors may induce stronger and more profound changes in the microbiota, while others such as short term antibiotic use or changes in diet may induce temporary shifts in the different bacterial populations and that they are able to, to a certain point, return to their original state (a term called resilience) [35].

As pointed out earlier, the gut microbiota plays an essential role in the development of a fully functional immune system in both mammals and teleosts [57,124,219]. Studies using germ-free mice reveal that these rodents have under-developed lymphoid tissues, defective T and B cell function, and low numbers of circulating CD4<sup>+</sup> T cells and antibody production, all of which can be restored by colonizing mice with microorganisms [28,123]. Similar findings have been described using germ-free zebrafish, which lack specific aspects of gut epithelium differentiation (specification and maturation) and proliferation, decreased protein macromolecule uptake and altered gut motility, all of which can be reversed by the introduction of intestinal microbiota [8,161,208]. In addition to helping shape the intestinal and systemic immune system, gut microbiota are essential for providing critical metabolic functions that cannot be accomplished by the host [145]. For example, some intestinal bacteria (obligate anaerobes) in mammals and teleosts are capable of degrading “non-digestible” complex carbohydrates (fiber) to produce short chain fatty acids (SCFA) such as acetate, butyrate and propionate (Fig. 2) [30,55]. These metabolites, rather than glucose, are the preferred energy substrate for colonic epithelial cells. In addition, certain bacteria (i.e., *Bacteroides*, *Bifidobacterium*, and *Enterococcus*) are capable of synthesizing vitamins as well as degrading different xenobiotics as it has been shown in humans [55,97,136].

The development of such dense and complex populations of microorganisms that reside so close to the gut requires that the intestinal immune system maintain tolerance to non-pathogenic/commensal bacteria. This is also true for teleosts whose immune system has to be able to coexist with commensal microorganisms in the gut, gills and skin [46]. Although the mechanisms responsible for tolerance have not been completely defined, there is good evidence to suggest that interactions between commensal bacteria and host mucosa induce highly regulated innate and adaptive immune responses [70,175]. For example, certain commensal bacteria such as segmented filamentous bacteria (SFB) have been shown to induce T helper 17 (Th17) cell differentiation in the small intestine, which protects the host from fungal and bacterial infections [5,76,77]. Interleukin 22 (IL-22), a Th17-derived cytokine, has been shown to enhance tight junctions in epithelial cells and increase mucin and antimicrobial protein production, all of which limit bacterial invasion into the lamina propria. Other

bacteria such as the *Clostridia* clusters IV, XIVa and XVIII and Bacteroidetes have been shown to induce immuno-regulatory T cells called regulatory T cells (Tregs; CD4<sup>+</sup>Foxp3<sup>+</sup> T cells). These T cells are known to suppress immune responses to self and bacterial antigens, promote epithelial repair, and promote tolerance to microbes [4,116,125,176]. In zebrafish, the intestinal microbiota also promotes the regulation of the innate immune responses to infections as reported by Galindo-Villegas et al. [51]. Furthermore, Rawls et al. demonstrated that the zebrafish gut microbiota is important for the expression of over 200 genes in the zebrafish intestine, many of which have also been observed in mice and are associated with epithelial proliferation, immune responses and nutrient metabolism [161].

### 3. Protection against invasion of unwanted neighbors

In mammals, the intestinal epithelial cell monolayer is composed of absorptive epithelial cells as well as specialized cells (e.g., goblet cells, Paneth cells) that are capable of secreting protective macromolecules and peptides. Immediately underlying the epithelium is the lamina propria which contains large numbers of plasma cells that produce IgA, a signature protective immunoglobulin of mucosal surfaces. Transcellular transport of IgA from the LP to the bowel lumen is mediated by the polymeric immunoglobulin receptor (pIgR), which is localized on the basolateral surface of epithelial cells [82]. Similar to mammals, the teleost intestine consists of three visually distinct anatomical divisions: the rostral intestinal bulb, the mid-intestine and the caudal intestine. In contrast with mammals, zebrafish do not contain a stomach [208]. Gene expression data in zebrafish suggest that the physiological functions of certain segments of its intestine are similar to mammals. Molecular characterization of the zebrafish transcriptome shows that the rostral and mid zebrafish intestine, where most metabolism, transport of nutrients, and energy metabolism take place, are functionally similar to the small intestine in mammals. In turn, the zebrafish caudal intestine is analogous to the mammalian large intestine [209]. It should be noted that crypts and Peyer's patches are absent in the zebrafish gut. The intestinal monolayer in zebrafish consists of three principal cell types: enterocytes, enteroendocrine cells, and goblet cells [103,129,216].

Invasion of pathogenic or commensal bacteria into the gut interstitium (i.e., LP) and their dissemination into the systemic circulation has the potential to produce devastating immune responses that could result in systemic inflammation, illness and possibly death. How does the gut limit and/or prevent the invasion of these unwanted neighbors? Essentially, the intestine has evolved four major levels of distinct but overlapping lines of defense that minimize contact of microbes with the intestinal epithelium, as well as eradicate microbes that manage to penetrate the epithelial barrier and gain access to the intestinal tissues [18,69]. The first line of defense in mammals is mediated by goblet cells, which are specialized epithelial cells that produce mucin, a highly glycosylated glycoprotein that polymerizes to form mucus. This macromolecular gel adheres to the apical surface of epithelial cells thereby providing a physical barrier that separates the epithelium from luminal bacteria [150]. The colon contains both an inner and outer layer of mucus. The inner layer of mucus is dense, highly polymerized, tightly adherent to the epithelial cell surface and relatively impermeable to the microbiota due to its resistance to most bacterial proteases [84]. The outer mucus layer is a loose, net-like polymer that occupies approximately 4 times the volume than the inner layer. It is not uncommon to observe some bacteria in the outer layer of mucus that may arise from limited proteolysis of the polymer that is mediated by the host as well as resident bacteria [82,83].

In contrast to the colon, the small intestine contains substantially smaller numbers of bacteria with the epithelium being covered by a single, loosely adherent layer of mucus. However, some pathogenic microorganisms have developed strategies to penetrate the mucus layers, allowing them to gain access to the epithelial cell surface through various strategies that vary among pathogens and commensals alike [38]. For instance, Yghl, an *Escherichia coli*-derived M60-like protease degrades mucus proteins, thus providing bacteria with access to the mouse small intestinal epithelium [110]. Pic, a secreted protease produced by *E. coli* and *Shigella flexneri*, degrades rat ileal mucus and stimulates compensatory mucus hypersecretion by goblet cells [140]. In addition, the food-born pathogen *Campylobacter jejuni* expresses JlpA surface lipoprotein adhesin, which allows for colonization of the human small intestine and colon [91]. Furthermore, the gastrointestinal pathogen *Salmonella enterica* penetrates cecal mucus using chemotaxis and flagella [193]. Under the highly acidic conditions of the stomach, *Helicobacter pylori* may reduce mucus viscosity via alkalization of the environment thereby allowing adhesion of these pathogens to the surface epithelium using SabA adhesin. It is hypothesized that  $\text{NH}_3$  that causes pH elevation is produced by *H. pylori* from the hydrolysis of urea [23,115]. The importance of mucus as a protective barrier has been directly demonstrated in genetically-engineered mice that have been rendered devoid of intact colonic mucus via deletion of the *Muc2* gene. These *Muc2*-deficient (*Muc2*<sup>−/−</sup>) mice exhibit growth retardation, enhanced bacterial invasion of colonic crypts and development of colonic inflammation by 5 weeks of age [202]. The absence of mucus may also synergize with other genetic defects such that mice devoid of both *Muc2* and the *IL-10* gene (*Muc2*<sup>−/−</sup>/*IL-10*<sup>−/−</sup> double deficient animals) develop exacerbated colitis when compared to colitis that develops in *Muc2*<sup>−/−</sup> or *IL-10*<sup>−/−</sup> animals [201]. These data suggest that chronic colitis in mucus-deficient mice may be due to the adhesion of bacteria to colonic epithelial cells (colonocytes) and invasion of these unwanted neighbors into the lamina propria where they activate innate and adaptive immune responses. In reality, the mechanisms for the induction of inflammation may be more complicated than simple adhesion/invasion of luminal bacteria as it is well-known that certain species of bacteria are capable of adhering to epithelial cells without invading the tissue or launching an intestinal inflammatory response. In fact, commensal bacteria–epithelial cell interactions appear to be required for homeostasis and for mediating tolerance to commensal bacteria [77,159]. The teleost intestinal tract is also endowed with a single protective layer of mucus. Zebrafish possess 5 genes that code expression of gel-forming mucin glycoproteins, which belong to two gene families: *Muc2* (*Muc2.1* and *Muc2.2* genes) and *Muc5* (*Muc5.1*, *Muc5.2* and *Muc5.3* genes). Sequencing of these genes revealed a great deal of homology to other vertebrates. According to gene sequencing data, the domain architecture of *Muc5.1* and *Muc5.2* zebrafish mucin proteins is typical for mammalian-secreted mucins [57,80]. The importance of intestinal mucus in zebrafish was demonstrated by Oehlers et al. who found that reduction of mucus secretion via administration of retinoic acid exacerbated enterocolitis induced by the hapten trinitrobenzene sulfonic acid (TNBS) in zebrafish larvae [146].

A second line of defense designed to limit the contact of bacteria with the intestinal epithelium is the secretion of different antimicrobial peptides (AMPs) by specialized epithelial cells (Fig. 2). In general, AMPs are restricted to the mucus with only minute amounts diffusing into the gut lumen [131]. Paneth cells, in spite of being a relatively rare cell type located in the intestinal crypts, are essential for limiting access of bacteria to the epithelium [11]. These specialized cells secrete  $\alpha$ -defensins (called cryptdins in mice) which bind to bacterial cell membranes and permeabilize them, resulting in death of the microorganism. In contrast to other

defensins, human  $\alpha$ -defensin 6 (HD6) does not destroy the bacterial membrane. Instead, it forms a nano-net of self-assembling fibrils on the surface of the bacterial cell thereby interfering with its ability to bind to the epithelium [14,149,210]. Of note, mice lack the HD6 analogue [183]. It appears that the secretion of cryptdins as well as cryptdin-related sequences (CRS) antimicrobial peptides is constitutive and does not depend upon the presence of luminal bacteria as it has been found in germ-free mice. In fact, production of cryptdins and CRS1C is increased only modestly in conventional mice challenged with *S. enterica* or *Listeria monocytogenes*. In contrast, CRS4C and bactericidal lectin Reg3 $\gamma$  are also produced constitutively by Paneth cells, but their secretion increases significantly following challenge with a pathogen [21,89,156]. Reg3 $\gamma$  is capable of killing Gram positive bacteria by binding to their membrane peptidoglycans and disrupting the bacterial cell wall [21]. In addition to Paneth cells, goblet cells secrete cysteine protease cathepsin K (Ctsk) [144,184]. Cathepsin K contributes to maintaining the normal composition of intestinal microflora as Ctsk<sup>−/−</sup> mice exhibit dysbiosis [184]. Although it has been demonstrated that other members of the cathepsin proteinase family are capable of lysing bacterial outer membranes [197], the mechanisms responsible for cathepsin K-mediated dysbiosis remain to be defined.

Another AMP produced by intestinal epithelial cells and inflammatory polymorphonuclear leukocytes (PMNs) is Lipocalin 2 (Lcn-2) [93]. This protein binds to the bacterial catecholate-type iron-chelating siderophores thereby preventing the acquisition of iron by bacterial cells, thus limiting bacterial growth [48,56]. Lcn-2 as well as heparin/heparin sulfate interacting protein (HIP/RPL29) expressed on the surface of mouse epithelial cells in the small intestine, are identical to those found in the mouse airway epithelium. These observations suggest that these proteins are part of the innate antibacterial defense of the epithelial surface in mammals [130,215]. Epithelial cell-derived AMPs are quite effective in limiting interactions between luminal bacteria and the epithelium; however, certain strains of bacteria have developed mechanisms whereby they may circumvent some of these antimicrobial properties. For instance, resistance to killing by host AMPs towards *Salmonella typhimurium* results from modifications of surface lipopolysaccharides (LPS) thereby reducing the efficacy of AMPs to bind to bacteria. In addition, *S. typhimurium* may secrete the iron-chelating molecule, salmochelin, is produced by glycosylation of another siderophore, enterobactin. Salmochelin is no longer bound by Lcn-2 and it is therefore, capable of supplying bacteria with iron [60,158].

Intracellular pattern recognition receptors (PRR) and the nucleotide-binding oligomerization domain receptor (NOD) proteins NOD1 and NOD2 are important regulators of mammalian innate immune responses which are expressed in Paneth cells of the intestinal epithelium as well as in many immune cell types [14,20]. Upon recognition of specific peptidoglycans (PGN) on the bacterial cell surface, NOD1 and NOD2 activate innate immunity in an NF- $\kappa$ B dependent manner [74]. In mice, it was shown that the lack of NOD2 expression causes intestinal dysbiosis [153]. Of interest, the human intracellular pathogen *L. monocytogenes* avoids recognition by NOD proteins due to the *N*-deacetylation of its surface peptidoglycan, providing protection from the host lysozyme and allowing it to replicate within host macrophages [16]. Zebrafish orthologs of both mammalian *Nod1* and *Nod2* genes have been identified and are highly homologous to their mammalian counterparts. Zebrafish *Nod2* gene splicing is shown to be similar to human *Nod2* [24,101,147]. The ability to control systemic infection is markedly reduced in *NOD1*<sup>−/−</sup> as well as *NOD2*<sup>−/−</sup> zebrafish larvae infected with *S. enterica*. Interestingly, the expression of intestinal *duox*, a gene that encodes for an enzyme that produces  $\text{H}_2\text{O}_2$ , is decreased in *NOD1*<sup>−/−</sup> larvae, but not in *NOD2*<sup>−/−</sup> larvae following challenge with *S. enterica*. This suggests that, unlike in

mammals, reactive oxygen species (ROS) production by DUOX in zebrafish is regulated by NOD1 and not by NOD2 [104,147]. Consistent with this hypothesis, increased bacterial load is observed in NOD1<sup>-/-</sup>, but not in NOD2<sup>-/-</sup> larvae [147]. In addition to DUOX, other AMPs have been identified in the teleost gut. For instance, Oehlers et al. reported expression of defensin  $\beta$ -like (*dfnb*) genes which are closely related to mammalian  $\beta$ -defensin genes, as well as expression of peptidoglycan recognition proteins (*pglyrp*) which exert their bactericidal properties against Gram positive bacteria [147]. Another group of investigators observed increased expression of cathepsin-like protein genes *ctsl1a* and *ctsl1b* as well as c-type lectin genes in the intestines of zebrafish infected by *Mycobacterium marinum* [203]. Brush border-associated alkaline phosphatase (*lap*) activity localized on zebrafish epithelial cells is also thought to be important for limiting microbe adhesion. Bacterial LPS from mucosa-associated bacteria is thought to induce the expression of Lap that dephosphorylates microbial LPS. Lap also downregulates myeloid differentiation primary response protein (MyD88)-dependent pro-inflammatory signaling in the intestine thereby preventing intestinal inflammation [7]. These data are consistent with the observation that MyD88<sup>-/-</sup> larvae display reduced survival as well as compromised responses to bacterial flagellin and LPS following challenge with *Edwardsiella tarda* and *S. typhimurium* [204].

A third line of defense by which vertebrates limit contact of luminal bacteria with their gut epithelium is by the synthesis and secretion of IgA. In mammals, approximately 70% of the total body production of IgA is induced by the intestinal microbiota [112,114]. IgA class switching by mucosal B cells is maintained by retinoic acid produced constitutively by resident dendritic cells (DC) as well as by transforming growth factor- $\beta$  (TGF- $\beta$ ) and other compounds provided by the intestinal microenvironment [113]. Numerous CD11c<sup>+</sup> DCs are located in the Peyer's patches and lamina propria of the small bowel. These professional antigen presenting cells continuously sample their immediate surroundings for bacteria and bacterial antigens. If present, DCs will phagocytize whole bacteria or antigens and migrate to the mesenteric lymph nodes (MLNs) where they present microbial peptides to naive B and T cells [43,114,142,164]. Following DC-mediated activation, B cells transform into plasma cells, leave the MLNs and enter the systemic circulation. These effector cells then home back to the intestinal lamina propria where they produce bacterial-specific immunoglobulin A (IgA) [68,83,138]. This bacterial specific immunoglobulin is then transported from the intestinal interstitium through epithelial cells via a specific pIgR receptor (see above) and into the gut lumen. Once IgA is deposited onto the luminal surface of the epithelium, it may bind to bacteria in close proximity to the epithelium, thereby preventing invasion of the microorganisms into the gut tissue. In addition, recent studies suggest that IgA coating of luminal bacteria facilitates their uptake by the M cells overlying gut-associated lymphoid tissue (e.g., MLNs, isolated lymphoid follicles), thereby limiting intestinal immune responses to the gut [152,185]. To date, IgA expression has not been detected in fish [22,167] and it is not clear if any particular immunoglobulin class in zebrafish may be considered a functional equivalent of mammalian IgA in mucosal immune responses. The *pIgR* gene expression in zebrafish gut has been confirmed in a recent study and unlike in mammals, zebrafish pIgR is composed of only two domains [98]. It is noteworthy that in another teleost, *Takifugu rubripes* (fugu), *pIgR* is capable of binding IgM in skin mucus [61]. In another study, the relatively primitive immunoglobulin IgT, also known as IgZ, is shown to function as a mucosal antibody and to be protective against intestinal parasites in rainbow trout [221].

The multiple lines of defense that mammals and teleosts have developed to protect against invasion of pathogenic and/or commensal bacteria are quite effective. Occasional breaches in the

epithelium may occur, allowing microorganisms to gain access to the intestinal lamina propria. When this occurs, vertebrates mobilize their fourth line of defense: innate and adaptive immunity. The vertebrate intestine is in actuality, the largest lymphoid tissue in the body which contains substantial numbers of dendritic cells, phagocytic leukocytes and lymphocytes [179]. Microbes which enter the gut lamina propria are rapidly phagocytized and killed by interstitial macrophages [186]. MyD88 signaling in these phagocytes is selectively downregulated insuring that intestinal macrophages produce relatively small amounts of pro-inflammatory cytokines and mediators while possessing potent bactericidal activity. This allows for the removal of unwanted microbes without fully activating an inflammatory response and consequent tissue injury [188,189]. These specific macrophages (called M2 macrophages) are crucial for limiting bystander tissue injury produced by innate immune responses. Intestinal M2 macrophages also contribute greatly to the repair of intestinal damage via their ability to induce the proliferation of epithelial cells to repopulate the injured epithelium [181]. The role of intestinal macrophages in teleosts has not been described in as much detail as their mammalian counterparts. However, their importance was demonstrated in zebrafish following intestinal pathogen *S. flexneri* infection. In mammals, it was shown that invading bacteria which penetrate the intestinal epithelium are phagocytized by indigenous macrophages and instead of being killed, ingested bacteria leave the phagosome causing macrophage cell pyroptosis [2]. In zebrafish, this leads to a dramatic decrease in macrophage numbers resulting in bacteremia and eventually death of the larvae [137]. This observation is especially valuable because it shows that zebrafish can be a relevant model for studying the pathogenicity of microorganisms such as *S. flexneri* that are not natural fish pathogens.

Another group of heterogeneous innate immune cells that reside within the gut and provide protection against invading microorganisms, as well as regulate adaptive immunity and wound healing are called innate lymphoid cells (ILCs). ILCs arise from hematolymphoid progenitor cells and may be categorized as cytotoxic ILCs which are represented by natural killer (NK) cells and helper-like ILCs that include ILC1, ILC2 and ILC3 [39]. NK cells and ILC1 produce large amounts of IFN- $\gamma$  that is used to protect mice against infection by certain bacteria such as *L. monocytogenes* [165]. ILC2 cells may also protect mice from certain helminths such as *Trichinella spiralis* via their production of the Th2 cytokine IL-13 [126]. What was considered a subset of NK cells (i.e., NK-22 cells) was shown to protect the host against bacterial invasion as well as maintain epithelial barrier integrity. These IL-22 producing ILCs, which are now considered ILC3, produce little or no IFN- $\gamma$  and express the retinoic-acid-receptor-related orphan receptor- $\gamma$ t (ROR $\gamma$ t) transcription factor [50,107,174,177]. It has been demonstrated that ILC3-derived IL-22 attenuates acute and chronic intestinal inflammation induced in lymphopenic mice (*Rag*<sup>-/-</sup> mice) by *Citrobacter rodentium* and by adoptive transfer of naive T cells, respectively [177,220]. Another important function of ILCs is anatomical containment of the intestinal microbiota [39,65,191]. Recent studies have demonstrated that immuno-depletion of ILCs resulted in the systemic dissemination of intestinal bacteria that was accompanied by multi-organ inflammation [191]. The loss of containment appeared to be due to the loss of ILC-derived IL-22 as the administration of IL-22 to ILC-depleted mice prevents the dissemination of commensal bacteria [191]. In follow up studies, Hepworth et al., showed that selective depletion of MHC ROR $\gamma$ t<sup>+</sup> ILCs expressing major histocompatibility complex II (MHC II) resulted in dysregulated commensal bacteria-dependent CD4<sup>+</sup> T cell responses that induced spontaneous and systemic dissemination of commensal bacteria resulting in multi-organ inflammation [65]. Taken together, these data suggest that intestinal homeostasis is mediated by the MHC II-dependent interaction between ILCs and CD4<sup>+</sup> T cells.

NK cells have currently not been described in zebrafish. It has been suggested that the recently described novel immune-type receptors (NITR) expressing cells in zebrafish and other teleosts may be functionally-equivalent to mammalian NK cells [78,218].

The adaptive immune cells (i.e., lymphocytes) represent another level of defense that synergizes with innate immune cells to maintain homeostasis, as well as protect the host from invasion of pathogenic microbes. One such group of lymphocytes that reside in large numbers within the epithelial monolayer are the intraepithelial lymphocytes (IELs). Natural IELs are either CD8 $\alpha^+$  or CD8 $\alpha^-$  and express TCR $\gamma\delta$  or TCR $\alpha\beta$  [27]. TCR $\gamma\delta$  IELs constitute approximately 60% of the total IELs of the small bowel and are generally thought to maintain epithelial integrity and limit immune activation [27]. Along with intestinal macrophages, TCR $\gamma\delta$  IELs are capable of producing keratinocyte growth factor (KGF) that mediates intestinal wound healing [26]. It has been shown recently that the ability of TCR $\gamma\delta$ -derived KGF to repair acute inflammatory injury in the gut is impaired in mice that lack an important ligand (semaphorin; CD100) for TCR $\gamma\delta$  activation [127]. Epithelial cells from CD100-deficient (CD100 $^{-/-}$ ) or KGF $^{-/-}$  mice exhibit significantly decreased proliferative rates in mouse models of experimental colitis. Due to the expression of the NKG2D NK receptor, TCR $\gamma\delta$  IELs are able to recognize the MHC I-like surface markers MICA and MICB expressed widely in the intestines of humans with IBD. NKG2D/MIC interaction is essential for cytotoxic killing of the enterocytes activated by allogeneic ligands [59,72]. TCR $\gamma\delta$  IELs stimulated by enterocytes may also respond to bacterial challenge by producing the AMPs Reg3 $\beta$  and Reg3 $\gamma$  [75]. In response to bacterial challenge, IELs migrate to enterocytes in contact with the pathogen, thus providing the host with immediate and essential protection against invading pathogen [41].

If intestinal bacteria evade the multiple layers of defense described above, they will eventually be eliminated by the adaptive immune response called cell mediated immunity. Whole bacteria and/or their antigens will be endocytosed by DCs within the LP. Antigen-loaded DCs will then travel from the LP to the MLNs via the efferent lymphatics. During this process, DCs will process and present the bacterial antigens in the context of their MHC II. Upon arrival to the MLNs, naïve CD4 $^+$  T cells will bind to the gut derived DCs via their TCR, whereupon the T cells become primed/activated and polarized and expand to produce larger numbers of antigen-specific Th1 and/or Th17 effector cells. It is during this time that T cells become “imprinted” via the increased expression of surface adhesion molecules such as CCR9, LFA-1, PSGL-1 and VLA-4. Once the effector cells have departed the MLNs and enter the systemic circulation, these imprinted homing proteins will direct migration of the T cells to the intestine where the levels of bacteria and antigens are the greatest [94]. Following extravasation into the gut LP, effector T cells will engage DCs as well as other antigen presenting cells (e.g., macrophages, B cells) that have also processed and presented the same bacterial antigens whereby the effector T cells become rapidly and fully activated to produce large amounts of IFN- $\gamma$ , IL-2, TNF- $\alpha$  and IL-17. Some of these cytokines will then activate intestinal macrophages to “help” in their phagocytosis and killing of bacteria. These inflammatory cytokines will also enhance the expression of endothelial cell adhesion molecules (e.g., E-selectin, ICAM-1, VCAM-1) thereby promoting the infiltration of additional phagocytic/myeloid leukocytes (e.g., PMNs, monocytes). Lugo-Villarino et al. have described a zebrafish myeloid cell that strongly resembles mammalian DCs morphologically and functionally and have MHC II expression [109]. Because innate and adaptive immune responses produce a number of cytotoxic species such as reactive oxygen and nitrogen metabolites, as well as secrete and activate different hydrolytic enzymes (e.g., metalloproteinases), the host must have ways to limit inflammatory tissue injury during these responses. This is accomplished by the action of thymic-

derived and peripheral induced regulatory Tregs [69,79,116,182]. In mice, the majority of Tregs express the Foxp3 transcription factor and CD25. Thymic-derived Tregs (tTregs) acquire expression of Foxp3 during their development in the thymus whereas induced Tregs (iTregs) are produced from conventional CD4 $^+$  T cells following their interaction with antigen-loaded DCs in lymphoid and nonlymphoid tissue [13,71]. Both populations of Tregs utilize IL-10 and/or TGF- $\beta$  to regulate innate and adaptive immune responses [148].

A list of cloned genes for teleost T cell-associated peptides analogous to those expressed in mammals includes  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  TCR subunits as well as CD3, CD4, CD8, CD28 and recombinae activated gene-1 (*Rag-1*) [9,17,57,196]. There are very few studies devoted to intestinal T cell function in zebrafish. The genes for zebrafish *Foxp3*, *Stat6*, and *T-bet* transcription factors have been cloned. *Foxp3* and *T-bet* zebrafish genes are identical to their human homologues, while the *Stat6* gene has some minor differences [132]. A gene for zebrafish IL-23 $\alpha$ , an essential component of IL-23, an interleukin required for Th17 cell differentiation in mammals, has been cloned recently as well [67]. Taken together, these data suggest that zebrafish may possess Th1, Th2, Th17 and Treg cells described in mammals. Mouse T cells, transduced with the zebrafish *Foxp3* gene, suppress activation of other T cells in *in vitro* co-culture assay [157]. Interestingly, two alternatively spliced transcripts have been discovered for the zebrafish *Foxp3* gene [132]. According to histologic staining data in rainbow trout, CD8 $^+$  cytotoxic T cells can make up more than half of the T cell population in the teleost's gut [196]. In sea bass, proliferation of T cells by allogeneic stimulation was reduced significantly by addition of cyclosporine A. It suggests that T cell proliferation in teleosts may be controlled by mechanisms similar to those reported for mammals [128].

#### 4. Loss of tolerance to the microbiota induces intestinal inflammation

The fact that vertebrates remain healthy despite the continuous presence of enormous numbers of potentially harmful microorganisms, attest to the remarkable ability of the intestinal immune system to maintain tolerance to nonpathogenic/commensal bacteria. It is becoming increasingly appreciated that the loss of tolerance to the commensal microbiota represents an important pathogenetic mechanism responsible for induction of intestinal inflammation [25,73,90,99,176,214]. Based upon their early work performed in rats and mice more than 20 years ago, Powrie and coworkers proposed that the chronic intestinal inflammation observed in patients suffering from IBD results from a dysregulated immune response to intestinal bacteria [3,79,154]. Evidence gathered over the past two decades suggests that IBD most likely arises from a complex interaction between genetic and environmental factors [73,90,99,214].

##### 4.1. Genetic susceptibility

To date, more than 160 different polymorphisms or susceptibility loci have been identified in patients with diagnosed IBD [25,87,90,206]. As one might expect, a substantial number of these loci common to both CD and UC are associated with innate and adaptive immunity. While the majority of the altered loci are associated with both CD and UC, genome wide associated studies (GWAS) have identified a smaller but significant number of polymorphisms that appear specific for CD. These include *NOD2*, autophagy related protein 16-like 1 (ATG16L1) and Immunity-Related GTPase Family M (*IRGM*) genes [87]. Furthermore, genes associated with epithelial barrier function have also been identified [87]. Because these genes express proteins that are

important for recognition and processing of intracellular bacteria as well as for limiting the invasion of intestinal bacteria into the gut lamina propria, one could envision a scenario whereby CD may arise from unrelenting bacterial infections [87]. In addition to defects in innate immunity, GWAS have reported several polymorphisms in genes that are crucial for the induction and regulation of adaptive immune responses such IL-17 and IL-23 signaling and IL-10/IL-10R interactions [87]. UC-specific susceptibility loci have also been described for genes responsible for HLA class II-mediated antigen presentation (e.g., *HLA-DQA1*), inflammatory mediator generation (e.g., *TNFRSF14*, *TMFRSF9*, *IL1R2*, *IL8Ra*, *IL7R*) and epithelial barrier function (e.g., *HNF4A*, *CHD1*, *LAMB1* genes) [87,92,206].

Much of our understanding of the immuno-pathogenesis of chronic gut inflammation has come from studies using mouse models of IBD [95,163,200]. Prior to the early 1990s, animal models of IBD required the oral or rectal administration of erosive chemicals to produce colonic mucosal damage that was followed by an acute self-limiting inflammatory response [95]. However, the publication of 4 paradigm-changing studies in 1993 altered dramatically the future of experimental IBD by demonstrating that mice rendered genetically-deficient in certain genes involved in immune regulation developed spontaneous and unrelenting colitis [100,135,154,173]. Since the publication of these ground-breaking studies, numerous other, genetically-engineered mouse models of chronic intestinal inflammation have been generated that continue to provide new and important information related to the immuno-pathogenesis of experimental IBD [85,95,163]. Although none of the mouse models completely recapitulate the clinical and immunohistopathological features of human disease, data obtained from several studies using chronic mouse models have revealed two important concepts that have served to direct basic and clinical research over the past two decades [95,163]. One basic concept that has come from these studies is that genetics play an important role in the pathogenesis of chronic inflammation. Indeed, numerous studies have shown that chronic inflammation develops in mice that possess one or more defects in genes that are involved in certain pathways related to innate and/or adaptive immune responses such as the *T-bet*<sup>-/-</sup> × *Rag-2*<sup>-/-</sup> ulcerative colitis (TRUC) [95,163,200].

Another important concept that has come from these mouse studies is that the “environment” (i.e., the intestinal microbiota) is required for induction and perpetuation of chronic gut inflammation in genetically-susceptible mice. Mice that are depleted of their microbiota using antibiotic administration or are delivered by caesarian section and raised under germ-free conditions develop little or no colitis [25,90,96,176]. In addition to mice, zebrafish are also being used as models for investigating the immunopathological mechanisms involved in intestinal inflammation [216]. These teleost models employ the use of chemical haptens [i.e., TNBS, oxazolone or irritants (dextran sodium sulfate (DSS))] to induce acute, self-limiting intestinal inflammation in larvae or adult fish [216]. Although not chronic in nature, these models have revealed several similarities to mouse-based models with respect to inflammation gene expression, leukocyte infiltration, intestinal injury and the requirement for intestinal bacteria [19,63,64,216]. Of note, some investigators have demonstrated that the knock down or deletion of genes that have been shown to be dysfunctional in human IBD or absent in mice that develop IBD, also increase susceptibility of zebrafish to bacterial infection [216].

#### 4.2. Environment: intestinal bacteria drive chronic gut inflammation

There is no question that genetics play an important role in the pathogenesis of IBD. However, genetics alone cannot fully account for the dramatic increase in IBD in modernized soci-

eties over the past 50 years. For example, concordance rates for IBD in identical twins is actually quite low with estimates of 30–35% and 10–15% for CD and UC, respectively [10]. In addition, it has been well-documented that countries/societies that have adopted a “Modernized” lifestyle have shown sharp increases in the incidence and prevalence of IBD over the past five decades [10,32,92,134,206]. Taken together, these studies suggest that alterations in intestinal microbiota produced by environmental factors may be responsible for the increased incidence in IBD throughout the world. Multiple studies have demonstrated that increased use (and abuse) of antibiotics, better environmental sanitation, increased personal hygiene, and alterations in diet and lifestyle dramatically alter the composition of intestinal microbiota [10,32,92,134,206]. Moreover, the rapid appearance of IBD in rural-based societies where these chronic diseases were once rare, have increased dramatically over the past 50 years following transition to more modernized communities. Taken together, these genetic and environmental observations support the hypothesis that environmental factors may play an important role in the induction of disease in genetically-susceptible individuals [10,118,134]. This is true not only for human disease but is also observed in mouse and teleost models of intestinal inflammation. As pointed out previously, many of the mouse models of chronic gut inflammation develop little or no disease in the absence of intestinal bacteria. In addition, changes in microbiota may alter dramatically the onset and/or severity of disease in these mice [95,96,163,200].

So, what exactly is the evidence that intestinal bacteria are an important pathogenetic factor that drives gut inflammation in genetically susceptible individuals? In fact, there is really only one study in humans that directly implicates intestinal bacteria in inducing IBD. Studies by Rutgeerts and coworkers showed that when the fecal stream of a segment of the small bowel that has been surgically resected (due to advanced CD) to form a neoterminal ileum was diverted, intestinal inflammation failed to develop [172]; however, when the fecal stream was restored inflammation rapidly appeared, suggesting a role for intestinal microbiota [34,172]. In addition to these direct studies, a number of indirect or association studies have been published demonstrating greater numbers of mucosa-, epithelia- and lamina propria-associated bacteria in histological samples obtained from inflamed vs. healthy intestine [25,118,119,122]. As mentioned previously, GWAS have revealed polymorphisms (mutations) in genes that are responsible for recognition and intracellular processing (and killing) of bacteria (i.e., *NOD2*, *ATG16L1* and *IRGM*) which are strongly associated with patients with CD [87]. Taken together, these data are consistent with the hypothesis that in addition to polymorphisms in genes associated with the adaptive immune system, defects in innate immune responses to intestinal bacteria may represent an important immunopathogenic factor that induces and perpetuates chronic intestinal inflammation [25,92,206]. The beneficial effects of certain antibiotics administered to patients with distal bowel inflammation is consistent with a role for the microbiota in disease pathogenesis [45].

Another important piece of indirect evidence implicating intestinal microbiota in the pathogenesis of human IBD is the observation that gut inflammation appears to always be accompanied by dramatic alterations in the luminal and mucosa-associated composition of intestinal bacteria, a situation called dysbiosis [176,192]. Numerous studies have reported marked dysbiosis in luminal (i.e., fecal) samples collected from humans with active IBD [54,99]. The large majority of these studies have used luminal/fecal samples and have consistently shown a decrease in bacterial diversity and abundance of bacteria within the Firmicutes and Bacteroidetes, whereas the abundance of certain Proteobacteria such as the Enterobacteriaceae (*E. coli*, AIEC) are increased [25,49,118,119,151,162,176]. However, a more recent study using large numbers of newly diag-

nosed, treatment-naïve patients with IBD, reports that from the trillions of possible bacteria that reside within the small and large bowel, alterations in abundance of only a select few groups of mucosa-associated bacteria were strongly correlated with presence and quality of disease [54]. This same study also emphasized that alterations observed in luminal/fecal microbial communities did not appear to correlate with disease status. These exciting studies have, for the first time, identified specific communities of mucosa-associated bacteria that may be very important in the induction and progression of chronic intestinal inflammation in genetically-susceptible individuals. Specifically, Gevers and coworkers observed increased abundance of certain pathobionts including *Enterobacteriaceae*, *Pasteurellaceae*, *Veillonellaceae* and *Fusobacteriaceae* as well as decreased abundance of “protective” *Erysipelotrichales*, *Bacteroidales* and *Clostridiales* [54]. Pathobionts are constituents of the healthy microbiota and are not considered classical pathogens; however these bacteria possess the potential to induce gut inflammation following disruption of the healthy microbiota [171].

Dysbiosis is also commonly observed in several mouse models of IBD [95,96]. Two more recent mouse models have extensively characterized this dysbiosis with the objective of defining the specific populations of pathobionts responsible for induction of disease. One model is the TRUC mouse model. This genetically-engineered mouse develops spontaneous, chronic and communicable colitis that is driven by the interaction between innate immune cells and the microbiota [53]. The fact that colitis is communicable and will develop in wild type mice when housed with TRUC mice suggests that there is an outgrowth of pathogenic bacteria in these mice [53]. Investigators have observed an overabundance of *Klebsiella pneumoniae* and *Proteus mirabilis* that correlated well with disease [52]. These two microbes are known to be increased in human IBD [52,53]. Another novel mouse model of IBD that requires the presence of intestinal bacteria and exhibits dysbiosis was created by Kang et al. [88]. These investigators bred dominant negative TGF $\beta$ RII (dnTGF $\beta$ RII) mice with IL-10R2<sup>-/-</sup> mice to generate dnTGF $\beta$ RII X IL-10R2<sup>-/-</sup> offspring which are referred to as dnKO mice. These offspring have defective TGF $\beta$  signaling in T cells and defects in IL-10R signaling in all tissues [88]. These dnKO mice develop an accelerated and unrelenting colitis that is abrogated by administration of broad spectrum antibiotics [88]. This same laboratory has also demonstrated that an isolate of *Bacteroides*, but not *Enterobacteriaceae* induces robust disease in dnKO but not in controls [15,66]. Taken together, these studies clearly demonstrate the need to assess the colitogenic potential rather than relying solely on 16S rRNA determinations. Despite the potentially important role that dysbiosis may play in the pathogenesis of IBD, it remains unclear whether dysbiosis is a cause or consequence of chronic gut inflammation. There are a few reports showing that dysbiosis may occur in healthy relatives of patients with IBD, suggesting that microbial alterations may precede disease, but little follow up of these family members has been documented [86]. In fact, it has been well-documented that products of the inflammatory reaction are effective at promoting dysbiosis [111]. Defining the role of dysbiosis in IBD may have to await more direct longitudinal studies that can only be accomplished in well-controlled mouse models of IBD. It should be noted that the intestinal microbiota has also been shown to be important for full expression of chemically-induced intestinal inflammation in zebrafish [19,63,64,216].

#### 4.3. Intestinal microbiota transplant: a novel therapeutic strategy for treating IBD

Great advances have been made over the past 30 years in understanding the immuno-pathological mechanisms responsible for the induction and perpetuation of IBD. Yet, only a handful

of pharmacologic and biologic agents are available to treat these debilitating inflammatory disorders. Furthermore, many of the current immunosuppressive small molecules available for patients may be toxic and the new-generation biologics can be expensive and require infusion in a physician's office. Thus, it is critical that additional, nontoxic and inexpensive therapies be developed to treat patients with IBD. Given the fact that there is now considerable experimental and clinical evidence demonstrating that the intestinal microbiota may act as a “trigger” to induce and perpetuate chronic gut inflammation in genetically-susceptible individuals, investigators have undertaken different avenues of research in an attempt to restore the dysbiotic gut microbiota that develops in patients with active IBD to a more typical, highly diverse community of microorganisms. A variety of different approaches have been used to manipulate the intestinal microbiota in IBD patients including the use of antibiotics, probiotics, and prebiotics. Manipulating the dysbiotic microbiota to decrease disease-producing pathobionts, while enhancing the numbers and metabolic activity of beneficial bacterial communities, has tremendous potential for therapeutic benefit. The therapeutic efficacy of antibiotic, probiotic and/or prebiotic administration in patients with IBD has not been proven in placebo-controlled, double blind, multicenter clinical studies. However, there are encouraging data suggesting that oral administration of ciprofloxacin and/or metronidazole may be effective in attenuating distal bowel inflammation in patients with CD [105,155,176,207]. Currently, antibiotic therapy is primarily used to treat complications such as fistulas and ulcers in IBD patients.

A great deal of interest has been generated following the publication of a recent clinical study that demonstrated that transplantation (i.e., colonization) of healthy fecal microbiota into patients with recurrent *Clostridium difficile* colitis essentially cured this recurrent and intractable infection [205]. Van Nood et. al., showed that 94% of the patients that received microbiota transplantation (FMT) had complete resolution of disease whereas only 31% of patients receiving current therapy (e.g., vancomycin) showed significant improvement [205]. These studies produced a great deal of excitement about the possible use of FMT in the treatment of IBD. Although a few open label case/cohort studies have reported tantalizing evidence suggesting a beneficial effect of FMT in the treatment of IBD, by and large, these studies are small, statistically underpowered and/or uncontrolled investigations. Two recent well-controlled clinical studies have been published that assessed the therapeutic efficacy of FMT in patients with UC. In one study, Moayyedi and coworkers reported, in a randomized, double blinded, placebo controlled study that a significantly greater percentage of patients receiving weekly FMT for 6 weeks achieved remission than those patients receiving a placebo (i.e., water; 24% vs. 5%) [133]. In the other study, Rossen et. al., treated one group of patients with active UC with healthy FMT or autologous FMT (infusion of patients own feces to serve as a placebo). These investigators found no significant difference in clinical and endoscopic remission between the two groups [170]. The reasons for the different outcomes in the two studies are not entirely clear at the present time. The two studies did, in fact, contain several major differences including location and frequency of the FMT infusion, concurrent use of anti-TNF therapy in one study, but not the other and possible donor microbial composition for efficacy [58,170]. Nevertheless, both of these studies reconfirm previous observations that FMT is well-tolerated. One potential caveat that has not been adequately addressed is whether the dysbiosis observed in patients with IBD is a cause or consequence of gut inflammation. If in fact dysbiosis is initially created by subclinical inflammation in genetically susceptible individuals leading to the expansion of disease-producing pathobionts such as adherent/invasive *Enterobacteriaceae*, the use

of FMT may not prove as effective as it would be if dysbiosis were the primary trigger of disease [212,213].

## 5. Conclusions

The vertebrate intestine is home to the vast majority of bacteria present on and within the body. The coexistence of mammals and teleosts with their gut microbiota is a continuously evolving process that provides numerous advantages to both host and bacteria. The intestinal microbiota which is now considered a virtual organ provides the host with the immunologic stimuli, nutrients and vitamins required for developing and maintaining health of the host. In turn, the host provides a physiological environment that is conducive for the steady-state bacterial colonization of the gut. The fact that mammal and teleost homeostasis is maintained in the presence of such large numbers of potentially dangerous microorganisms is testament to the remarkable ability of the intestinal immune system in these land and aquatic animals to maintain tolerance to commensal bacteria while mounting effective immune responses to pathogenic microbes. The inability of the intestinal immune system to maintain tolerance to the intestinal microbiota is thought to be a major immuno-pathogenic mechanism responsible for the induction of IBD in humans. Indeed, there is an accumulating literature suggesting that Crohn's disease and/or ulcerative colitis results from a dysregulation of immune responses toward intestinal bacteria in genetically-susceptible individuals.

## Conflict of interest

The authors declare that there is no conflict of interest.

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# Application of comparative transcriptional genomics to identify molecular targets for pediatric IBD

Kai Fang<sup>1\*</sup>, Matthew B. Grisham<sup>2</sup> and Christopher G. Kevil<sup>3,4\*</sup>

<sup>1</sup> Division of Digestive Diseases, Inflammatory Bowel Disease Center, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

<sup>2</sup> Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, Lubbock, TX, USA

<sup>3</sup> Department of Pathology, Louisiana State University Health Sciences Center, Shreveport, LA, USA

<sup>4</sup> Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, LA, USA

## Edited by:

Pietro Ghezzi, Brighton and Sussex Medical School, UK

## Reviewed by:

Manuela Mengozzi, Brighton and Sussex Medical School, UK

Wenzhong Xiao, Massachusetts General Hospital and Harvard Medical School, USA

## \*Correspondence:

Kai Fang, Division of Digestive Diseases, Inflammatory Bowel Disease Center, David Geffen School of Medicine at UCLA, 675 Charles E Young Drive S, MRL 1519, Los Angeles, CA 90095-7019, USA  
e-mail: kfang@mednet.ucla.edu;  
Christopher G. Kevil, Department of Pathology, Louisiana State University Health Science Center Shreveport, 1501 Kings Highway, Shreveport, LA 71103, USA  
e-mail: ckevil@lsuhsc.edu

Experimental models of colitis in mice have been used extensively for analyzing the molecular events that occur during inflammatory bowel disease (IBD) development. However, it is uncertain to what extent the experimental models reproduce features of human IBD. This is largely due to the lack of precise methods for direct and comprehensive comparison of mouse and human inflamed colon tissue at the molecular level. Here, we use global gene expression patterns of two sets of pediatric IBD and two mouse models of colitis to obtain a direct comparison of the genome signatures of mouse and human IBD. By comparing the two sets of pediatric IBD microarray data, we found 83 genes were differentially expressed in a similar manner between pediatric Crohn's disease and ulcerative colitis. Up-regulation of the chemokine (C–C motif) ligand 2 (CCL2) gene that maps to 17q12, a confirmed IBD susceptibility loci, indicates that our comparison study can reveal known genetic associations with IBD. In comparing pediatric IBD and experimental colitis microarray data, we found common signatures amongst them including: (1) up-regulation of CXCL9 and S100A8; (2) cytokine–cytokine receptor pathway dysregulation; and (3) over-represented IRF1 and IRF2 transcription binding sites in the promoter region of up-regulated genes, and HNF1A and Lhx3 binding sites were over-represented in the promoter region of the down-regulated genes. In summary, this study provides a comprehensive view of transcriptome changes between different pediatric IBD populations in comparison with different colitis models. These findings reveal several new molecular targets for further study in the regulation of colitis.

**Keywords: interferon regulatory factor, chemokines, chitinase 3-like 1, transcription, bioinformatics**

## INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the two major forms of inflammatory bowel disease (IBD). The incidence rate of pediatric CD in the US is 43 per 100,000 and 28 per 100,000 for pediatric UC (1). As recently reported, the incidence and prevalence of pediatric IBD is rising in both developed and developing countries (2). Growth retardation poses a significant threat to the quality of life of 15–40% of children and adolescents with IBD (3). Although environmental factors, microbes in the gastroenterological tract, genetic susceptibility, and immune system dysfunction have been implicated, the etiology of pediatric IBD remains incompletely understood.

During the development of IBD, the colon tissue changes its genome transcription in response to pathological conditions, which is a result of dysregulated interaction between the immune system and enteric bacteria. The common feature of UC and CD inflamed tissue genome transcription provides new clues for pediatric IBD treatment. Although the microarray assay has been performed on pediatric IBD, there is no comprehensive genome transcription analysis for pediatric UC or CD. Here, we performed transcriptome analysis using two sets of pediatric IBD microarray

data (4, 5), T-cell transfer colitis model microarray data (6), and dextran sodium sulfate (DSS)-induced colitis microarray data (7) generated from our laboratory and deposited in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database. Network and promoter analysis was performed to identify differentially expressed genes in the inflamed colon tissue from pediatric IBD patients versus experimental animal models. Comparison between pediatric IBD and experimental colitis microarray data revealed the similarly expressed genes and over-represented transcription factor binding sites (TFBS) in the promoter regions of the dysregulated genes.

## MATERIALS AND METHODS

### PEDIATRIC IBD MICROARRAY DATASETS

To get a comprehensive view of the pediatric IBD genome transcription profile, two sets of pediatric IBD microarray data were selected from NCBI. Those two sets of microarray data were obtained by using Affymetrix GeneChip Human Genome HG-U133 plus 2.0 arrays that provide the most comprehensive coverage of transcribed human genome and contain probes for approximately 22,634 genes. The microarray data were generated from

pediatric colon in healthy controls, colon only CD, and colon only UC. The dataset GSE10616 contained data from 11 control samples, 14 CD samples, and 10 UC samples (4); the dataset GSE9686 contained data from 8 control samples, 11 CD samples, and 5 UC samples (5). Colon RNA was isolated from biopsies obtained from patients and healthy controls at diagnosis. The pediatric Crohn's Disease Activity Index (PCDAI) and Pediatric Ulcerative Colitis Clinical Activity Index (PUCAI) were used to assess the clinical severity of the IBD sample.

### GENESIFTER ANALYSIS

Two sets of pediatric IBD microarray data were uploaded to GeneSifter software<sup>1</sup> and normalized for comparison by Robust Multichip Average (RMA) method. The gene expression difference threshold was set to 2 with no upper limit. Data were analyzed with a Student's *t*-test followed by a Benjamini and Hochberg post test to limit false discovery rates, as we previously reported (7).

### INGENUITY PATHWAY ANALYSIS

To see the relationship between differentially expressed genes, the selected genes identified as dysregulated in pediatric CD and pediatric UC microarray data were then imported to IPA<sup>2</sup> for network analysis. Genes that were related to each other in biological functions and/or diseases were organized into networks according to the Ingenuity Pathways Knowledge Base (IPKB). IPKB is a database derived from the data mining of the expression of and functional relationships between molecules; this information was extracted from published papers found in NCBI PubMed, Medline, and several other databases.

### CIS-REGULATORY ELEMENTS ANALYSIS

To identify common properties of promoter regions of differentially expressed genes, the Affymetrix gene ID of identified genes in pediatric UC and CD were uploaded to the cREMaG system<sup>3</sup> (8). The sequence upstream the transcription start site (TSS) is 5000 base pairs, and the downstream sequence of TSS is 1000 base pairs. Promoter sequences were scanned with TFBS matrices obtained from the JASPAR database and the public release of the TRANSFAC database using the TFBS BioPerl module (9, 10). The top 10 of the most over-represented binding sites were selected for comparison analysis.

### COMPARISON BETWEEN PEDIATRIC IBD AND EXPERIMENTAL COLITIS MICROARRAY DATA

By directly comparing differential gene expression between human and mouse inflamed colon tissue, we assessed the similarity between human and mouse colitis. The dysregulated gene in DSS-colitis (GEO data base accession number GSE22307) and T-cell transfer colitis model (accession number GSE27302) were divided into eight classes according to the genes expression trends. In the DSS-colitis model, there were 1609 genes that were significantly altered during the colitis development, with 501 progressively up-regulated genes and 173 progressively down-regulated genes

(7). In the T-cell transfer colitis model, there were 1775 gene expressions that were significantly changed, with 341 progressively up-regulated genes and 361 progressively down-regulated genes (6). The two sets of microarray data were obtained by using the same platform, Mouse Genome 430 2.0 Array (Affymetrix), which provided the most comprehensive annotated coverage of the mouse genome, composing of over 34,000 well-characterized mouse genes. The genes whose expression progressively changed were correlated with inflammation development and were selected for promoter binding sites analysis. The over-presented promoter binding sites were further compared with the over-presented binding sites obtained from pediatric IBD array data.

## RESULTS

### GENESIFTER ANALYSIS PEDIATRIC IBD MICROARRAY DATA

Analysis of GSE 9686 pediatric CD microarray data showed that 242 genes were differentially expressed, 173 genes had up-regulated expression, while 69 genes had down-regulated expression. Analysis of the GSE 10616 pediatric CD microarray data showed that there were 298 genes differentially expressed (the expression of 209 genes were up-regulated and 89 genes were down-regulated). After comparing two sets of pediatric CD microarray data, we found the expression of 167 genes was similarly changed. Among those 167 genes, 117 genes were up-regulated (Table S1 in Supplementary Material), and 50 gene expressions were down-regulated (Table S2 in Supplementary Material).

In GSE9686 pediatric UC microarray data, there were 3860 genes differentially expressed (1717 genes were up-regulated, and 2143 genes were down-regulated). While in GSE10616 pediatric UC, there were 1826 genes differentially expressed (1122 genes were up-regulated and 704 genes were down-regulated). After comparing the two sets of pediatric UC data, we found that 1071 genes were similarly up-regulated (Table S3 in Supplementary Material), and 736 genes were down-regulated (Table S4 in Supplementary Material).

After comparing the data in Tables S1 and S3 in Supplementary Material, we found that there were 65 genes up-regulated in pediatric CD and pediatric UC, as shown in **Table 1** and **Figure 1A**. By comparing Tables S2 and S4 in Supplementary Material, we found that there were 18 genes down-regulated in pediatric CD and pediatric UC, as shown in **Table 2** and **Figure 1B**.

Of the up-regulated genes, seven were from the CXC chemokine family: CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL9, and CXCL11, which are the key components of the cytokine–cytokine receptor interaction pathway. CXCL1 is expressed by epithelial cells, macrophages, and neutrophils (11, 12) and has neutrophil chemoattractant activity (13). CXCL2 is secreted by macrophages and monocytes and is a chemoattractant for polymorphonuclear cells, leukocytes, and hematopoietic stem cells (11, 14, 15). CXCL5 is expressed in eosinophils and stimulates the chemotaxis of neutrophils (16). CXCL6 is a chemoattractant for neutrophils (17). CXCL9 is an interferon (IFN)-dependent CXC chemokine, which plays a pro-inflammatory role and has been found to be expressed at high levels in UC tissue (18). CXCL11 is a chemoattractant for activated T cells (19).

Of the down-regulated genes, four were solute carrier genes: SLC16A9, SLC17A4, SLC23A3, and SLC3A1. The functions of

<sup>1</sup><http://www.genesifter.com>

<sup>2</sup><http://www.ingenuity.com>

<sup>3</sup><http://www.cremag.org>

**Table 1 | Similarly up-regulated genes in pediatric IBD.**

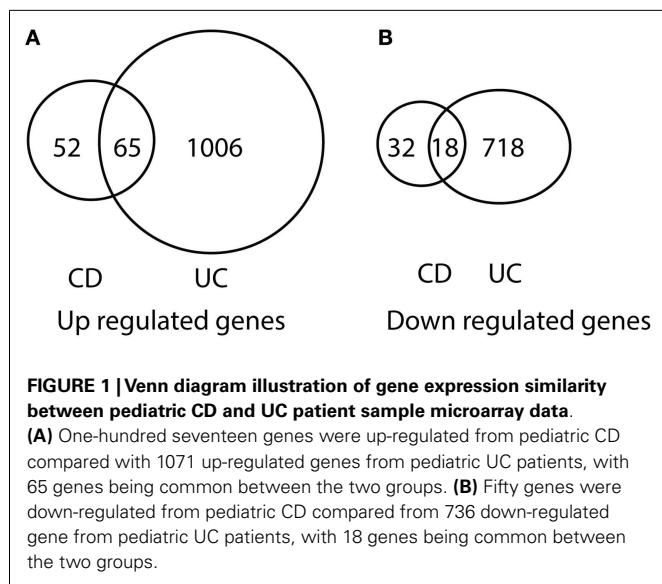
| Gene ID   | Gene identifier | GSE9686 CD | GSE10616 CD | GSE9686UC | GSE10616UC |
|-----------|-----------------|------------|-------------|-----------|------------|
| ACSL4     | NM_022977       | 2.52       | 2.38        | 5.46      | 4.19       |
| ADM       | NM_001124       | 2.19       | 2.25        | 3.70      | 3.17       |
| ALDH1A2   | AB015228        | 2.22       | 2.93        | 11.29     | 8.36       |
| APCDD1    | N48299          | 2.3        | 2.28        | 3.45      | 2.94       |
| C4BPA     | NM_000715       | 2.01       | 2.50        | 10.31     | 5.94       |
| CCL2      | S69738          | 2.37       | 2.40        | 3.16      | 2.73       |
| CCRL1     | NM_016557       | 2.04       | 2.13        | 5.18      | 3.13       |
| CDH11     | D21254          | 2.52       | 2.34        | 6.21      | 4.08       |
| CFB       | NM_001710       | 2.25       | 2.27        | 3.44      | 3.19       |
| CFI       | BC020718        | 2.04       | 2.10        | 6.36      | 3.38       |
| CH25H     | NM_003956       | 2.01       | 2.02        | 3.66      | 2.99       |
| CHI3L1    | M80927          | 6.15       | 5.72        | 33.46     | 21.81      |
| CLDN1     | AF101051        | 2.17       | 2.54        | 8.57      | 4.59       |
| COL1A2    | NM_000089       | 2.02       | 2.51        | 5.01      | 4.08       |
| COL4A1    | NM_001845       | 2.03       | 2.09        | 4.27      | 3.65       |
| COL6A3    | NM_004369       | 2.07       | 2.35        | 4.24      | 3.97       |
| CXCL1     | NM_001511       | 5.60       | 4.66        | 18.35     | 13.43      |
| CXCL11    | AF002985        | 6.82       | 5.61        | 14.19     | 12.26      |
| CXCL2     | M57731          | 3.35       | 3.39        | 10.29     | 9.44       |
| CXCL3     | NM_002090       | 2.89       | 3.27        | 9.44      | 8.29       |
| CXCL5     | AK026546        | 6.04       | 6.49        | 44.64     | 22.12      |
| CXCL6     | NM_002993       | 4.84       | 5.97        | 30.42     | 18.36      |
| CXCL9     | NM_002416       | 4.70       | 3.23        | 4.13      | 3.95       |
| CYP27B1   | NM_000785       | 2.03       | 2.19        | 2.35      | 2.22       |
| CYR61     | NM_001554       | 3.11       | 2.98        | 3.69      | 3.94       |
| DERL3     | AI655697        | 2.25       | 2.11        | 5.50      | 3.96       |
| DUOX2     | NM_014080       | 11.06      | 9.73        | 29.14     | 25.35      |
| DUOXA2    | AI821606        | 2.24       | 3.79        | 14.00     | 13.6       |
| EMR2      | NM_013447       | 2.55       | 2.54        | 5.01      | 4.20       |
| FCGR1A    | X14355          | 2.82       | 2.39        | 2.88      | 2.61       |
| FCRL5     | AF343662        | 2.47       | 2.53        | 6.18      | 4.60       |
| FN1       | X02761          | 2.08       | 2.07        | 2.15      | 2.32       |
| HSD11B1   | NM_005525       | 2.68       | 2.57        | 4.12      | 3.84       |
| IGKV1D-13 | AVV408194       | 3.19       | 2.52        | 6.96      | 4.87       |
| IGLV1-44  | U96394          | 3.63       | 3.26        | 15.57     | 9.51       |
| IVD       | AF043583        | 3.00       | 3.08        | 11.48     | 7.58       |
| KDELR3    | NM_006855       | 2.38       | 2.50        | 5.09      | 4.13       |
| LCN2      | NM_005564       | 3.11       | 3.27        | 7.12      | 6.18       |
| LOXL2     | NM_002318       | 2.23       | 2.31        | 4.77      | 3.78       |
| LPL       | BF672975        | 2.19       | 2.73        | 6.44      | 4.22       |
| MMP1      | NM_002421       | 5.44       | 6.69        | 41.57     | 22.03      |
| MMP10     | NM_002425       | 3.71       | 4.86        | 44.39     | 34.11      |
| MMP3      | NM_002422       | 11.65      | 10.85       | 66.73     | 49.68      |
| MS4A2     | NM_000139       | 2.33       | 2.12        | 3.73      | 2.96       |
| NEBL      | NM_006393       | 2.24       | 2.29        | 3.94      | 3.24       |
| NIACR2    | NM_006018       | 4.90       | 4.04        | 7.94      | 8.03       |
| NID1      | BF940043        | 2.39       | 2.44        | 5.00      | 3.61       |
| NOS2      | L24553          | 2.12       | 2.02        | 4.07      | 3.54       |
| NTN3      | AF103529        | 3.64       | 2.91        | 9.44      | 6.10       |
| PCDH7     | NM_002589       | 2.07       | 2.16        | 7.21      | 4.27       |
| PCSK1     | NM_000439       | 3.25       | 4.54        | 11.66     | 8.33       |
| PHLDA1    | AA576961        | 2.89       | 2.91        | 9.53      | 6.48       |

(Continued)

**Table 1 | Continued**

| Gene ID | Gene identifier | GSE9686 CD | GSE10616 CD | GSE9686UC | GSE10616UC |
|---------|-----------------|------------|-------------|-----------|------------|
| PSAT1   | BC004863        | 3.49       | 2.49        | 9.33      | 5.50       |
| S100A8  | NM_002964       | 6.09       | 5.66        | 21.7      | 17.55      |
| SEC24D  | NM_014822       | 2.35       | 2.19        | 4.85      | 3.49       |
| SGMS1   | AI377497        | 2.07       | 2.17        | 5.15      | 3.56       |
| SLC6A14 | NM_007231       | 7.11       | 10.32       | 56.81     | 51.22      |
| SOCS3   | AI244908        | 2.36       | 2.55        | 4.49      | 4.88       |
| SOD2    | W46388          | 2.29       | 2.31        | 3.86      | 3.03       |
| SPINK4  | NM_014471       | 6.02       | 5.83        | 17.04     | 11.85      |
| TFPI2   | L27624          | 3.68       | 3.22        | 10.00     | 7.01       |
| TIMP1   | NM_003254       | 2.29       | 2.34        | 5.44      | 4.89       |
| TMEM158 | BF062629        | 2.46       | 3.06        | 8.01      | 5.83       |
| TMEM45A | NM_018004       | 2.40       | 2.67        | 9.10      | 5.01       |
| TYRP1   | NM_000550       | 2.34       | 2.79        | 6.58      | 5.63       |

The two set of pediatric microarray data are downloaded from NCBI Gene Expression Omnibus (NCBI GEO) database. Values are mean fold change normalized to control.



those down-regulated genes related to the transport of amino acids (20), monocarboxylate (21), glutamate (22), sodium-phosphate (23), and ascorbic acid (24).

#### INGENUITY ANALYSIS OF PEDIATRIC IBD MICROARRAY DATA

Those genes differentially expressed in pediatric CD and UC were uploaded to Ingenuity software for network analysis. Those differentially expressed genes in pediatric CD inflamed colon tissue were organized into eight networks. The molecules in each network and their top functions are listed in Table S5 in Supplementary Material. Those differentially expressed genes in pediatric UC inflamed colon tissue were organized into 25 networks, and the molecules in each network and their top functions are listed in Table S6 in Supplementary Material.

**Figure 2** shows the first network of pediatric CD inflamed colon tissue differentially expressed genes with their cell-to-cell signaling

functions and their interactions, as they relate to gastrointestinal and hepatic system disease. As shown in **Figure 2**, the transcription of nine chemokine genes was up-regulated, and those genes indirectly react with the NF- $\kappa$ B complex. **Figure 3** shows the network 2 differentially expressed genes in pediatric CD inflamed colon tissue, which is composed of 15 up-regulated genes with functions related to connective tissue and genetic disorders. MMP-1 and MMP-3 are located in the center of pediatric CD network 2. ChI3L1 is also implicated in this network through its indirect reaction with IGFBP5.

**Figure 4** shows the first network of pediatric UC with functions related to cellular movement and signaling. Pediatric UC network 1 is mainly composed of eleven G-protein-coupled receptors, which were all up-regulated. Transcription of eight members of the collagen family was up-regulated as shown in **Figure 5**, with functions related to connective tissue. Interestingly, ChI3L1 was in the center of the pediatric UC network 2 (**Figure 5**), and ChI3L1 indirectly interacts with COL16A2 and TNC.

#### PROMOTER ANALYSIS OF PEDIATRIC IBD MICROARRAY DATA

Using the CREMaG system, we identified over-presented TFBS in the differentially expressed genes. The over-presented TFBS of differentially regulated genes in pediatric CD is shown in Tables S7–S10 in Supplementary Material. TFBS over-presented in pediatric UC differentially expressed genes are shown in Table S11 in Supplementary Material (for up-regulated genes) and in Table S12 in Supplementary Material (for down-regulated genes). The fold-difference in TFBS frequency was computed by dividing the observed TFBS number by the background number.

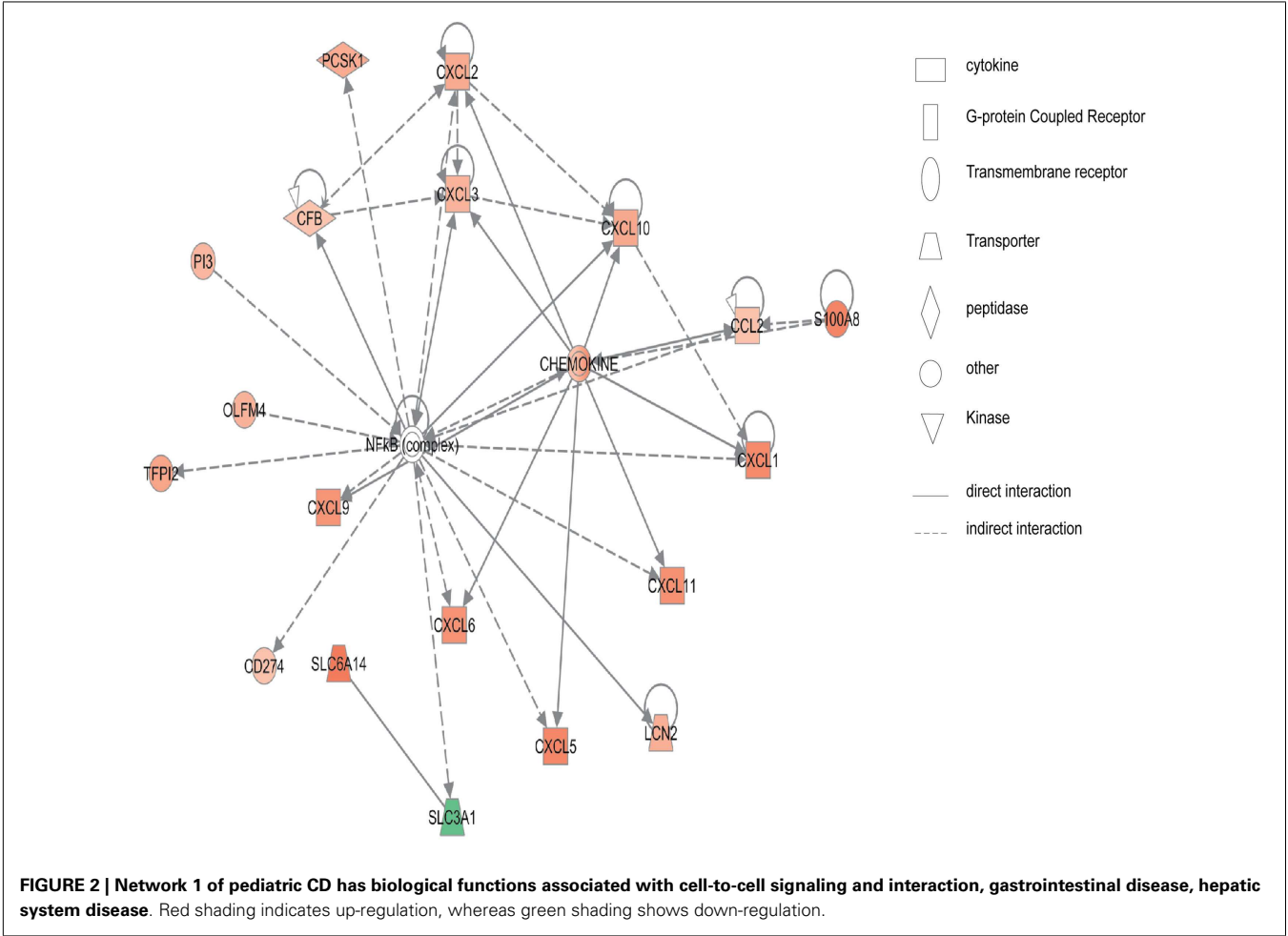
By comparison, we found that there were six promoter sequences (RELA, NF- $\kappa$ B, IRF2, Evi1, and IRF1) that were over-presented in genes that were up-regulated in pediatric IBD. There were six TFBS (Lhx3, MEF2A, HNF1A, Nobox, NR2F1, and Foxa2) that were over-presented in the genes that were down-regulated in pediatric IBD-inflamed colon tissue.

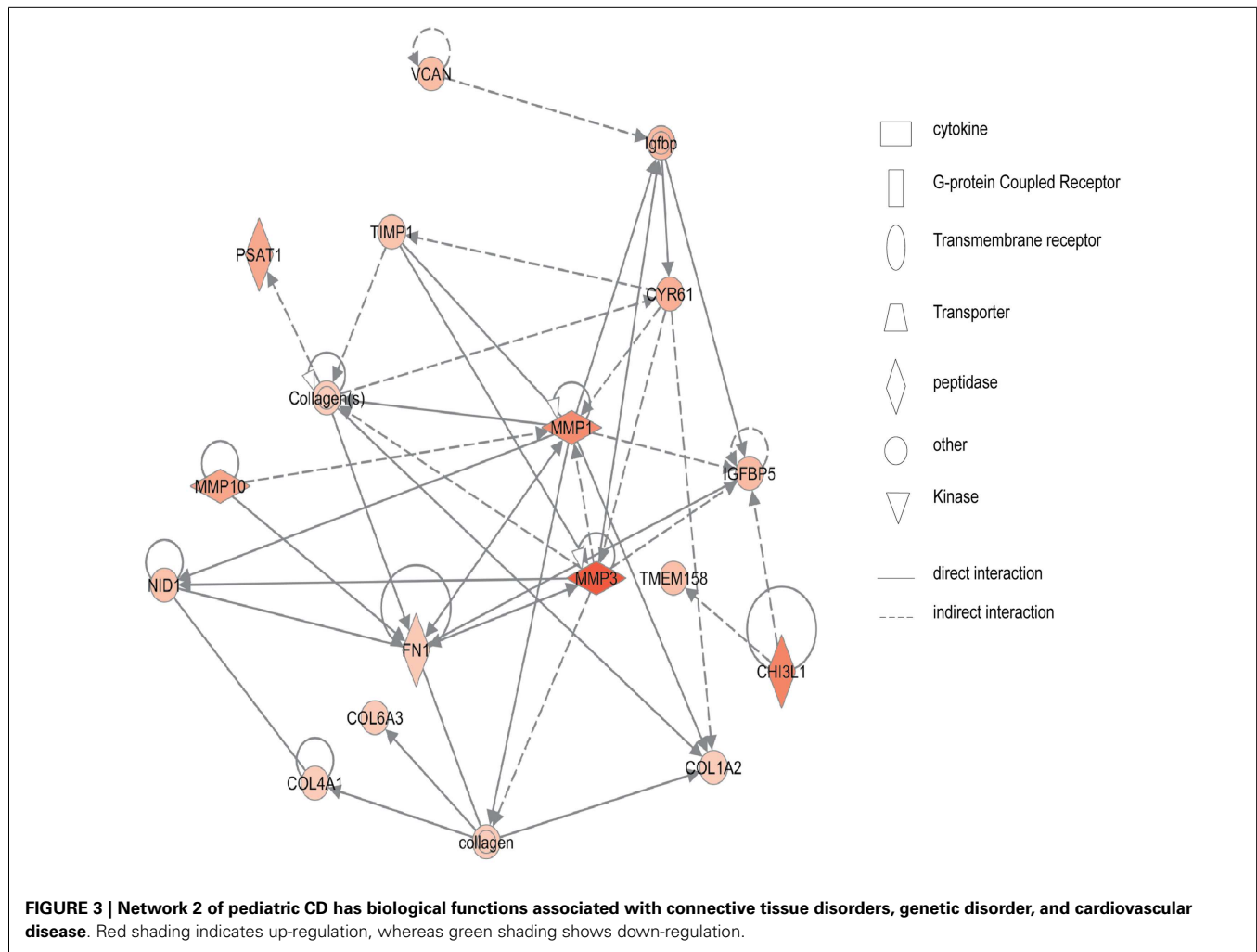
In the pediatric CD microarray data analysis, the NF- $\kappa$ B binding sequence was over-presented in the inflammatory-related

Table 2 | Similarly down-regulated genes in pediatric IBD.

| Gene ID   | Gene identifier | GSE9686 CD | GSE10616 CD | GSE9686UC | GSE10616UC |
|-----------|-----------------|------------|-------------|-----------|------------|
| ABCB1     | AF016535        | −2.13      | −2.02       | −4.98     | −4.04      |
| ABCG2     | AF098951        | −4.93      | −3.09       | −8.52     | −9.93      |
| APOBEC3B  | NM_004900       | −2.09      | −2.05       | −2.78     | −2.80      |
| AQP8      | NM_001169       | −4.57      | −3.85       | −28.58    | −36.55     |
| KRT12     | NM_000223       | −2.53      | −2.22       | −4.34     | −3.69      |
| LOC389023 | AI499651        | −2.07      | −2.81       | −3.56     | −3.60      |
| LOC643008 | BF478120        | −2.02      | −2.00       | −4.50     | −2.58      |
| MEP1B     | NM_005925       | −5.08      | −3.82       | −6.37     | −8.09      |
| PCDH21    | AI825832        | −2.15      | −2.24       | −6.15     | −3.70      |
| PHLPPL    | AB023148        | −2.13      | −2.01       | −4.29     | −3.99      |
| PLA2G12B  | BF939574        | −2.29      | −2.36       | −2.80     | −3.73      |
| PRAP1     | AA502331        | −2.93      | −2.33       | −3.99     | −4.65      |
| SGK2      | AI631895        | −2.6       | −2.92       | −4.76     | −4.35      |
| SLC16A9   | BG401568        | −4.05      | −3.08       | −6.67     | −4.65      |
| SLC17A4   | NM_005495       | −2.64      | −2.58       | −8.08     | −6.66      |
| SLC23A3   | AI263078        | −2.61      | −2.27       | −5.24     | −3.98      |
| SLC3A1    | M95548          | −3.11      | −2.43       | −3.25     | −4.76      |
| WSCD1     | AB011095        | −2.17      | −2.05       | −2.62     | −2.73      |

The two set of pediatric microarray data are downloaded from NCBI Gene Expression Omnibus (NCBI GEO) database. Values are mean fold change normalized to control.





genes, such as CCL2, CXCL10, CXCL2, CXCL3, CXCL6, CXCL9, and in other up-regulated genes in the pediatric CD-inflamed colon tissue. The network analysis of pediatric CD (Figure 2) also showed that NF- $\kappa$ B regulates chemokine gene expression. Not surprisingly, in the pediatric UC microarray data promoter analysis, the NF- $\kappa$ B binding site was also shown to be over-represented in the promoter region of the up-regulated genes, as it is well known that NF- $\kappa$ B plays a pivotal role in the expression of inflammatory mediators. The promoter region of 89 up-regulated genes (ICAM-1, COL1A1, WNT5A, CXCL5, IL-1B, CXCL2, IL-6, IL-11, and others) in pediatric UC has the NF- $\kappa$ B binding site.

#### COMPARISON OF PEDIATRIC IBD AND EXPERIMENTAL COLITIS MICROARRAY DATA

The dysregulated KEGG pathway of GSE9686 pediatric UC and GSE10616 pediatric UC is shown in Tables S13 and S14 in Supplementary Material. The over-represented TFBS of progressively up-regulated or down-regulated genes in the T-cell transfer colitis model and the DSS-colitis model are shown in Tables S15–S18 in Supplementary Material.

Comparison of differentially expressed genes in pediatric IBD and progressively up-regulated or down-regulated genes in experimental colitis is shown in Table 3 and Figures 6 and 7. The comparison of the over-presented promoters in the differentially expressed genes is shown in Table 4. Among them, the cytokine–cytokine receptor pathway was dysregulated in all the four sets of microarray data. CXCL9 and S100A8 were up-regulated in all the four sets of microarray data. The expression of S100A8 was also found to be up-regulated in the trinitrobenzene sulfonic acid (TNBS)-induced colitis rat model and T-cell-mediated colitis in SCID mice (25, 26).

Promoter analysis provided the possible common regulatory mechanism of the expression of the dysregulated genes. As shown in Table 4, the IRF1 and IRF2 binding sites were over-represented in the up-regulated genes in pediatric IBD and experimental colitis. The HNF1A and Lhx3 binding sites were over-presented in the down-regulated genes in the four sets of microarray data. HNF1A is a transcription factor that regulates the expression of cytokine-driven C-reactive protein, which is a clinical marker of inflammation (27). Lhx3 is a transcription factor that is required for pituitary and motor neuron development (28).

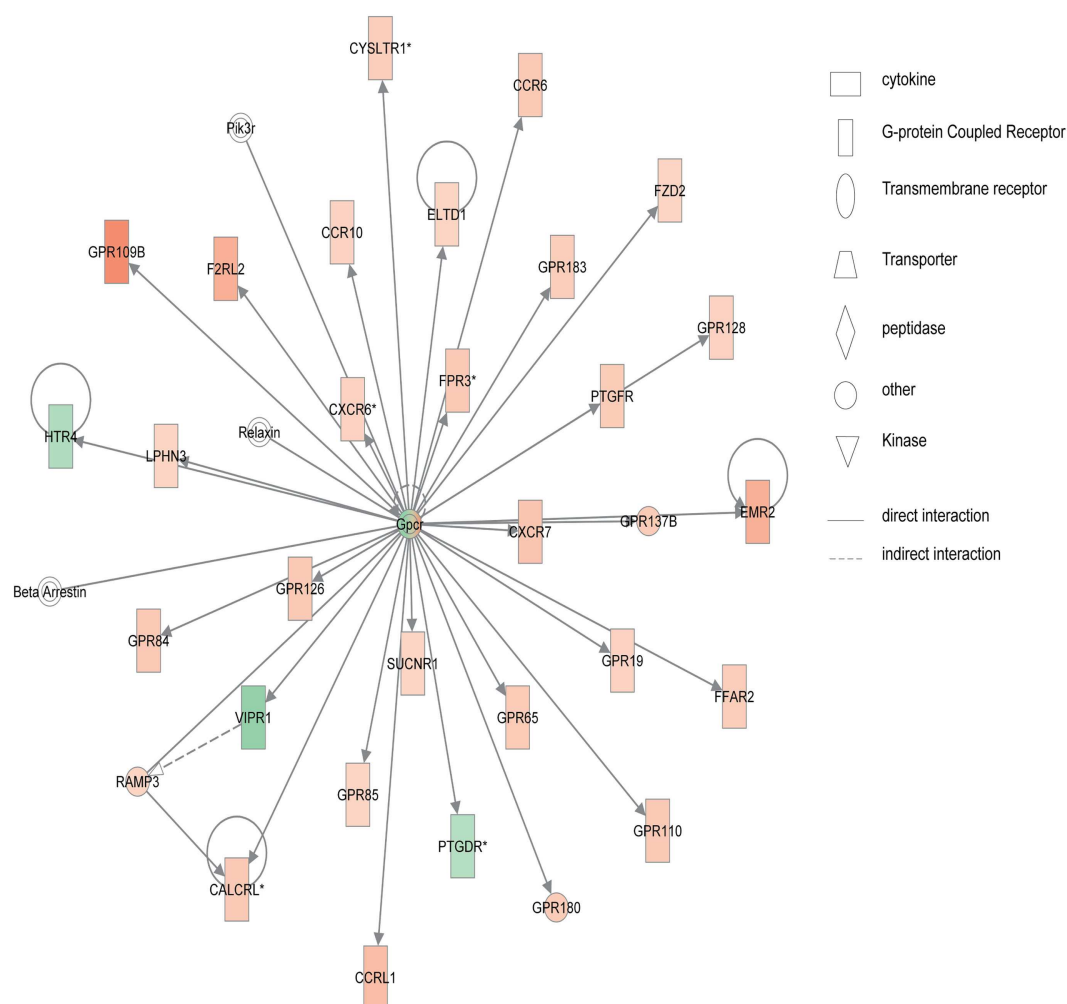


**FIGURE 4 | Network 1 of pediatric UC has biological functions associated with cellular movement, cell signaling, and nucleic acid metabolism.** Red shading indicates up-regulation, whereas green shading shows down-regulation.

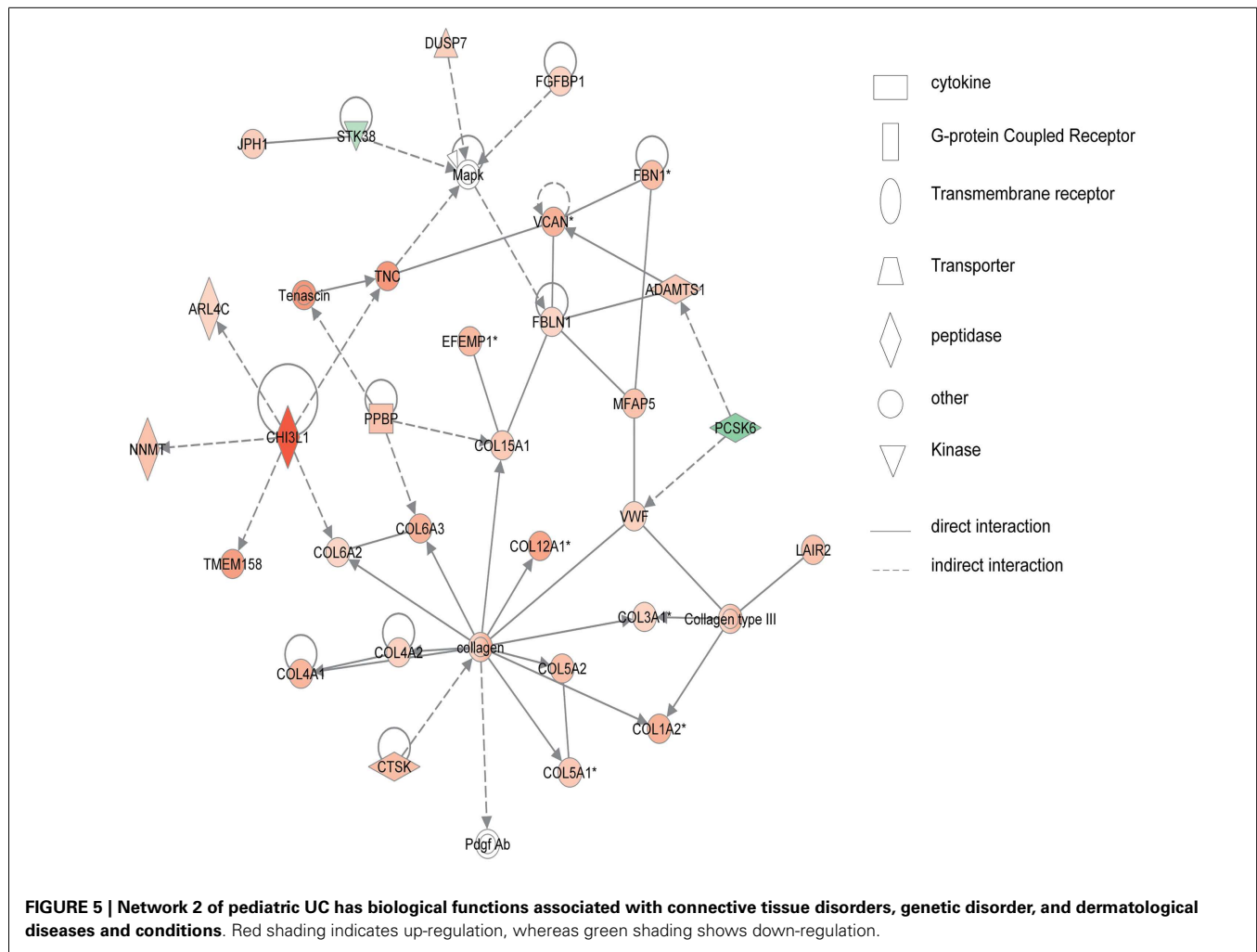
As shown in **Table 3**, cytokine–cytokine receptor interaction pathway is dysregulated in pediatric UC, CD, T-cell transfer, and DSS-colitis model. While chemokine signaling pathway is dysregulated in pediatric UC, CD, and T-cell transfer colitis model, but not in DSS-colitis model. Additionally, NF- $\kappa$ B binding site is over-presented in the promoter region of up-regulated genes in pediatric CD, UC, and T-cell transfer colitis, but not in DSS-colitis model (**Table 4**). Thus, the comparison of dysregulated KEGG pathway (**Table 3**) and the over-represented TFBS (**Table 4**) showed that the T-cell transfer colitis model was better than the DSS-induced colitis model at simulating pediatric IBD; however, the DSS-colitis model was more similar to pediatric UC than pediatric CD, as the DSS model has more common dysregulated pathways and molecules (**Table 3**) and over-represented TFBS in the dysregulated genes with pediatric UC than CD (**Table 4**).

## DISCUSSION

Our microarray analysis revealed that chitinase 3-like 1 (cartilage glycoprotein-39, CHI3L1) was up-regulated in pediatric IBD

samples. CHI3L1 has the ability to enhance the adhesion and internalization of bacteria in epithelial cells (29). *In vivo*, neutralizing CHI3L1 with an antibody suppresses DSS-induced colitis, and this neutralization dramatically decreases bacteria adhesion and invasion of epithelial cells. It has been demonstrated that CHI3L1 expression is up-regulated in epithelial cells under inflammatory conditions. CHI3L1 also activates Akt signaling in epithelial cells through its chitin binding motif, and increases secretion of IL-8 and TNF- $\alpha$  in a dose-dependent manner (30). Fecal CHI3L1 levels are positively correlated with pathology score (31). Serum concentration of CHI3L1 is also elevated in IBD patients (32). Thus, CHI3L1 might be selected as both a target and a marker of pediatric IBD.

Cysteine-rich, angiogenic inducer 61 (CYR61 or CCN1) was up-regulated in pediatric IBD. It has been demonstrated that CCN1 up-regulates pro-inflammatory gene transcription, such as TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and IL-12b in mice macrophages (33). This induction results from CCN1 direct activation of NF- $\kappa$ B and increased TNF- $\alpha$  synthesis. CCN1 supports macrophage



adhesion through integrin  $\alpha\text{M}\beta 2$  and syndecan-4. Because mice lacking CCN1 cannot develop *in utero*, involving vascular defects in the placenta, CCN1 is also related with vasculogenesis during embryogenesis (34). Moreover, CCN1 construct transfected mice showed increased angiogenesis in colon tissue (35). Together, these suggest that CCN1 might be a unique target to treat pediatric IBD through inhibition of pro-inflammatory gene expression and angiogenesis.

Chemokine (C–C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 or small inducible cytokine A2, was up-regulated in pediatric inflamed colon tissue. CCL2 attracts monocytes, memory T cells, and dendritic cells to sites of tissue injury, infection, and inflammation (36, 37). Interestingly, CCL2 is located in the confirmed CD and UC susceptibility loci 19q12 (38, 39). Increased expression of CCL2 (Table 1) in pediatric inflamed colon tissue supports the idea that CCL2 might be one of the causal genes of pediatric IBD. Interestingly, this has been demonstrated by nanomolar concentrations of CCL2 stimulating inflammatory responses of monocytes and effector T cells, whereas picomolar CCL2 exerts a global suppressive effect on T-cell trafficking into inflamed lymph

nodes (40), as confirmed by picomolar levels of CCL2 ameliorating TNBS- and DSS-induced colitis (41). Thus, before targeting CCL2 for IBD therapy more information is needed regarding its dose effect on human colon tissue inflammation responses.

One major difference of genome transcription in adult IBD versus pediatric IBD colon tissue is that there is fewer common dysregulated genes found in adult IBD. Feng et al. found that 25 genes were up-regulated and 18 genes were down-regulated in adult IBD inflamed colon tissue (42). We found that 65 genes were up-regulated and 18 genes were down-regulated in pediatric IBD colon tissue. Compared with those two studies, CXCL2 and CXCL3 were both up-regulated in pediatric and adult IBD, and only ABCB1 was down-regulated in pediatric and adult IBD. This comparison suggests that there is a large difference between pediatric and adult IBD patients. Additionally, both studies show that there is a difference between CD and UC genome transcription patterns, which suggests that CD and UC have distinctive pathogenesis.

Promoter analysis provided potential targets at the transcription factor level. NF- $\kappa$ B transcription factors are comprised of

**Table 3 | Common dysregulated pathways and genes.**

|                               | Dysregulated pathways                                                                                                                                                                                                      | Genes up-regulated                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | Genes down-regulated                                                           |
|-------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| T-cell model vs. pediatric CD | Cytokine–cytokine receptor interaction, chemokine signaling pathway, Leishmaniasis, Chagas disease, asthma, malaria, NOD-like receptor signaling pathway                                                                   | CXCL1, CXCL10, CXCL2, CXCL5, CXCL9, DUOX2, GBP1, IL1B, MMP3, PSAT1, S100A8, TGM2                                                                                                                                                                                                                                                                                                                                                                                                              | ABCB1                                                                          |
| T-cell model vs. pediatric UC | Cytokine–cytokine receptor interaction, chemokine signaling pathway, cell adhesion molecules (CAMs), hematopoietic cell lineage, Leishmaniasis, malaria, Alzheimer's disease, Huntington's disease, graft-vs.-host disease | ARL4C, CTSC, CD274, CDC6, CCR1, CXCL1, CXCL2, CXCL9, CSF2RB, C1R, C2, DUOX2, EGR2, PSAT1, PYHIN1, RUNX2, S100A8, S100A9, SLPI, SRGN, STAT1, SLAMF8, SLC7A11, SNX10, SOCS1, TGM2, WARS, UBD, ZC3H12A                                                                                                                                                                                                                                                                                           | ABCB1, CHKA, DENND1B, FGFR3, HOXB5, HSD3B2, RBM25, SCIN, VSIG2                 |
| DSS model vs. pediatric CD    | Cytokine–cytokine receptor interaction, Toll-like receptor signaling pathway, ECM-receptor interaction                                                                                                                     | ACSL4, APCDD1, ALDH1A2, CCL2, CXCL10, CXCL9, CHI3L1, HSD11B1, IGFBP5, LCN2, MMP10, MMP3, PCDH7, S100A8, SOCS3                                                                                                                                                                                                                                                                                                                                                                                 | ANK3, AQP8, PHLPL                                                              |
| DSS model vs. pediatric UC    | Cytokine–cytokine receptor interaction, complement and coagulation cascades, cell adhesion molecules (CAMs), Toll-like receptor signaling pathway, ECM-receptor interaction, hematopoietic cell lineage                    | ACSL4, APCDD1, ALDH1A2, CTSK, CD300A, CD86, CCL2, CCL3, CCR1, CXCL9, CHRDL2, COL18A1, CSF2RB, CLEC7A, CRISPLD2, CYP1B1, EDNRA, EDNRB, EPHA3, EMP3, GPR84, GJA1, IGFBP5, ICAM-1, IFIT3, IL-11, IL33, IL-6, LMCD1, LCN2, LHFPL1, LUM, LCP2, MMP10, MMP12, MMP2, MMP3, NR2F1, OLFML2B, OLFML3, OSMR, PDE4B, PLXNC1, KCNJ8, PCOLCE, PSMB9, PCDH7, PYHIN1, QK1, RSPO3, S100A8, S100A9, SAMS1, SLPI, SH3PXD2B, STAT1, SLAMF8, ST8SIA4, SOCS3, STX11, TUBB6, TNFSF11, TNFSF11B, TWIST1, VGLL3, WISP1 | ABAT, ACVR1C, ANK3, AQP8, ABCC6, MALAT1, PHLPL, RBM25, SLC26A2, SLC26A3, TRPM6 |

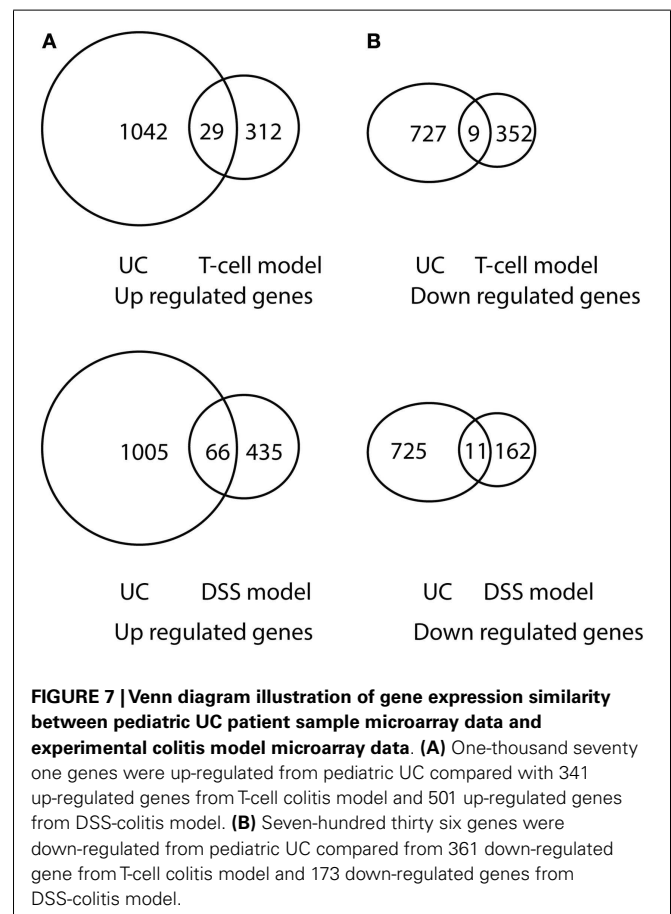
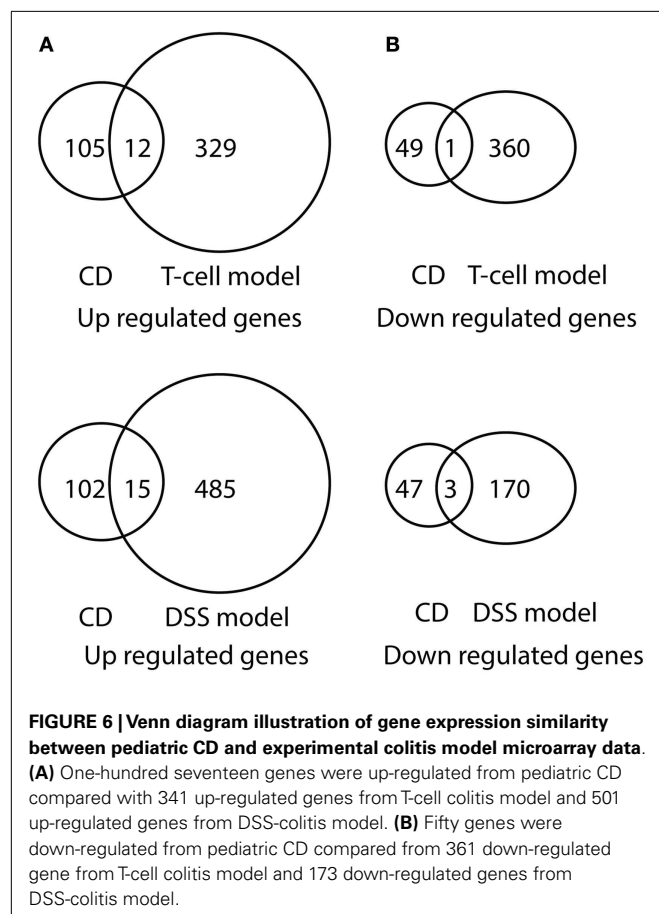


**Table 4 | Comparison promoter between animal model and pediatric IBD.**

|                               | Over-represented in up-regulated genes                  | Over-represented in down-regulated genes |
|-------------------------------|---------------------------------------------------------|------------------------------------------|
| T-cell model vs. pediatric CD | IRF1<br>IRF2<br>NF- $\kappa$ B<br>RELA<br>ELF5          | Lhx3<br>MEF2A<br>HNF1A<br>Nobox          |
| T-cell model vs. pediatric UC | IRF1<br>IRF2<br>NFYA<br>RELA<br>FOXF2<br>NF- $\kappa$ B | HNF1A<br>Lhx3<br>Pax4<br>MEF2A<br>Nobox  |
| DSS model vs. pediatric CD    | IRF2<br>IRF1                                            | Lhx3<br>HNF1A<br>Foxa2                   |
| DSS model vs. pediatric UC    | IRF2<br>Pax4<br>IRF1<br>FOXF2                           | HNF1A<br>Lhx3<br>Pax4<br>IRF2<br>Foxa2   |

five family members in mammalian cells: RelA (p65), RelB, c-Rel, p50/p105 (NF- $\kappa$ B1), and p52/p100 (NF- $\kappa$ B2). Those members form homo- or hetero-dimers of NF- $\kappa$ B complexes to regulate the expression of a variety of genes (43). Interestingly, Stronati et al. found that nuclear NF- $\kappa$ B and the binding activity of NF- $\kappa$ B to a consensus DNA sequence were significantly increased in the inflamed mucosa of patients, compared to controls (44). In IBD patients, the increased NF- $\kappa$ B expression in mucosal macrophages is accompanied with an increasing capacity of these cells to produce and secrete TNF- $\alpha$ , IL-1, and IL-6 (45). Many of the established immunosuppressive drugs for IBD, like corticosteroids, play anti-inflammatory roles, at least partly via the inhibition of the NF- $\kappa$ B activity (46). The fact that the NF- $\kappa$ B binding sites are over-represented in the T-cell transfer colitis model, combined with the fact that the NF- $\kappa$ B binding sites are over-represented in pediatric IBD inflamed colon tissue suggests that NF- $\kappa$ B likely plays an important role in pediatric IBD. In agreement with this idea, NF- $\kappa$ B has been extensively studied, and different ways to block NF- $\kappa$ B have been evaluated for IBD treatment. Unfortunately, due to significant side effects and liver toxicity, optimal ways to block NF- $\kappa$ B to treat IBD has not been realized (47).

Interferon regulatory factor-1 and -2 (IRF1 and IRF2) are transcription factors that regulate expression of inflammatory-related genes, but are primarily identified as transcription factors which regulate the human IFN- $\alpha/\beta$  gene (48). Interestingly, our promoter analysis showed that binding site of IRF1 and IRF2 are over-represented in the pediatric IBD up-regulated genes.

Additionally, Clavell et al. found increased expression of IRF1 in lamina propria mononuclear cells from patients with CD (49). Compared with wild-type mice, production of TNF- $\alpha$  and IFN- $\gamma$  in IRF1<sup>-/-</sup> mice is greatly impaired (50). Mice with a target mutation in IRF2 (IRF2<sup>-/-</sup>) exhibit significant inhibition of IL-12, IL-12R, IFN- $\gamma$ , IL-1  $\beta$ , and IL-6 expression (51). It has been demonstrated that IRF2 recruits the NF- $\kappa$ B transcription factor into the nucleus via physical interaction, which enhances TNF- $\alpha$ -induced NF- $\kappa$ B transcription (52). Thus, IRF1 and IRF2 have the potential to be selective and potentially effective targets for the treatment of both experimental colitis and pediatric IBD.

In conclusion, we performed pediatric IBD transcriptome analysis and its cross-species comparison with experimental colitis models. Identification of common dysregulated gene expression profiles, over-represented transcription binding sites, and related transcription factors controlling dysregulated gene expression changes reveal several molecular targets that serve as novel pathways for further study and potential therapy for pediatric IBD.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2015.00165/abstract>

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# Use of Humanized Mice to Study the Pathogenesis of Autoimmune and Inflammatory Diseases

Iurii Kobozev, PhD,\* Yava Jones-Hall, DVM, PhD,<sup>†</sup> John F. Valentine, MD,<sup>‡</sup> Cynthia Reinoso Webb, BS,\* Kathryn L. Furr, MS,\* and Matthew B. Grisham, PhD\*

**Abstract:** Animal models of disease have been used extensively by the research community for the past several decades to better understand the pathogenesis of different diseases and assess the efficacy and toxicity of different therapeutic agents. Retrospective analyses of numerous preclinical intervention studies using mouse models of acute and chronic inflammatory diseases reveal a generalized failure to translate promising interventions or therapeutics into clinically effective treatments in patients. Although several possible reasons have been suggested to account for this generalized failure to translate therapeutic efficacy from the laboratory bench to the patient's bedside, it is becoming increasingly apparent that the mouse immune system is substantially different from the human. Indeed, it is well known that >80 major differences exist between mouse and human immunology; all of which contribute to significant differences in immune system development, activation, and responses to challenges in innate and adaptive immunity. This inconvenient reality has prompted investigators to attempt to humanize the mouse immune system to address important human-specific questions that are impossible to study in patients. The successful long-term engraftment of human hematolymphoid cells in mice would provide investigators with a relatively inexpensive small animal model to study clinically relevant mechanisms and facilitate the evaluation of human-specific therapies in vivo. The discovery that targeted mutation of the IL-2 receptor common gamma chain in lymphopenic mice allows for the long-term engraftment of functional human immune cells has advanced greatly our ability to humanize the mouse immune system. The objective of this review is to present a brief overview of the recent advances that have been made in the development and use of humanized mice with special emphasis on autoimmune and chronic inflammatory diseases. In addition, we discuss the use of these unique mouse models to define the human-specific immunopathological mechanisms responsible for the induction and perpetuation of chronic gut inflammation.

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**Key Words:** hematopoietic stem cells, fetal thymus, fetal liver, interleukin-2 common gamma receptor, NK cells, NOD mice, SCID mice, human immune system, rheumatoid arthritis, graft versus host disease, diabetes, allograft rejection

Animal models of disease have been used extensively by the research community over the past several decades as surrogates to better understand the pathogenetic mechanisms responsible for the induction of different diseases, to assess toxicity, determine pharmacokinetics of different compounds, and to evaluate the efficacy of novel therapeutic agents. Surprisingly, retrospective meta-analyses of preclinical intervention studies using animal models of a variety of different acute and chronic inflammatory diseases, such as stroke, sepsis, diabetes, multiple

sclerosis, and rheumatoid arthritis, report that only a small fraction of promising preclinical interventions and novel therapeutics translate to clinical efficacy.<sup>1–7</sup> A cursory search of PubMed using the keywords mouse and colitis identifies >5800 studies that have been published using mouse models of the inflammatory bowel diseases (IBD; Crohn's disease, ulcerative colitis). Of these, hundreds of studies report significant anti-inflammatory effects of numerous small molecules, biologics, genetic alterations, or immune manipulations in these models of IBD. Yet, very few of the potential "targets" or therapeutic interventions identified in this voluminous literature have been taken to the next level and evaluated in clinical studies. In fact, of the more than 50 novel small molecules, biologics and cell-based therapies that have been reported to be effective in preclinical animal studies and have been or are currently being evaluated in dozens of phase I–III clinical studies, only monoclonal antibodies directed against TNF (i.e., infliximab, adalimumab, certolizumab, golimumab) or  $\alpha_4(\beta_7)$  integrins (i.e., natalizumab, vedolizumab) have been shown to be effective in clinical studies and approved for treatment of patients with IBD (reviewed in Ref. 8; <http://www.clinicaltrials.gov>). The reasons for the disconnect between preclinical studies and therapeutic efficacy have not been clearly delineated;

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From the \*Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, Lubbock, Texas; <sup>†</sup>Department of Comparative Pathobiology, Purdue University College of Veterinary Medicine, West Lafayette, Indiana; and <sup>‡</sup>Department of Internal Medicine, Gastroenterology, Hepatology and Nutrition, University of Utah, Salt Lake City, Utah.

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Reprints: Matthew B. Grisham, PhD, Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, 3601 4th Street, STOP 6591, Lubbock, TX 79430-6591 (e-mail: [matthew.grisham@ttuhsc.edu](mailto:matthew.grisham@ttuhsc.edu)).

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however, several possible factors are thought to be involved, including (1) the use of animal models that do not adequately mimic the chronic immunopathology of human IBD, (2) the use of inbred strains of mice as surrogates for heterogeneous human populations, (3) differences in intestinal microbiota, (4) flawed experimental design and/or data analyses, and (5) publication bias.<sup>1-7,9</sup> In addition to these shortcomings in the design and evaluation of preclinical studies, a particularly troubling situation has emerged over the past few years that has garnered a great deal of attention by funding agencies and the publishing community: the inability of academic and industry investigators to reproduce published studies demonstrating therapeutic efficacy of novel small molecules and biologics in animal models of disease.<sup>2,10-15</sup>

One potential strategy for improving the bench-to-bedside transition for promising therapeutics is to identify and use the most immunologically relevant mouse models of IBD and pharmacologic strategies that most closely mimic the clinical situation.<sup>1</sup> However, even with more rigorous standardization of preclinical studies, we are faced with the reality that mice are not humans, and thus, the immunopathogenetic mechanisms observed in mouse models of chronic inflammation may not necessarily recapitulate those for human disease. It is well known that the structure and function of the mouse immune system is, in many instances, significantly different from humans.<sup>16,17</sup> For example, mice possess relatively few lymph nodes that are grouped together in small numbers of “chains.” Humans, however, possess larger numbers of lymph nodes that are organized into complex chains that drain relatively small areas of tissue when compared with mouse.<sup>16</sup> In addition to peripheral lymphoid structures, the structure and function of mouse bronchus-, nasal- and gut-associated lymphoid tissues may differ dramatically from those of the human depending on its anatomical location.<sup>16</sup> Given the structural differences in the immune systems between species, it is not surprising to learn that certain functions of the immune system are also quite different in mice when compared with humans. It is well appreciated that >80 major differences exist between mouse and human immunology that are involved in immune system development, activation, and responses to immune challenges.<sup>17</sup> Major differences between the 2 species include hematopoietic activity of the spleen, circulating levels of lymphoid and myeloid leukocytes, innate immune mechanisms (e.g., Toll-like receptors [TLRs], NOD-like receptors, defensins), T-cell signaling pathways, B-cell function, IgA production and isotype, intestinal intraepithelial and lamina propria cell composition, gut-associated dendritic cell and natural killer T-cell subsets, and inducible nitric oxide synthase.<sup>9,17-21</sup> Indeed, these differences may account, in part, for the lack of consensus as to whether mouse models of inflammation can be used as surrogates for human inflammatory diseases. Although one recent study suggests that genomic responses in mouse models of inflammation do not replicate those observed in human diseases,<sup>22</sup> another equally compelling study concluded that gene expression patterns in mouse models closely mirror human inflammatory responses.<sup>23</sup>

This inconvenient reality, coupled to the fact that animal models have been underwhelming in their ability to predict clinical trial outcomes in a variety of human diseases, reinforces the need to develop small animal models that more closely recapitulate human immunity. The development of mice with a fully functional human immune system through the long-term engraftment of the human hematolymphoid cells (e.g., hematopoietic stem cells [HSCs], peripheral blood mononuclear cells [PBMCs]) would provide investigators a preclinical model to more accurately define disease pathogenesis and facilitate the evaluation of human-specific therapies *in vivo*. This approach has been successfully used for mechanistic and intervention studies in models of human-specific infectious diseases (e.g., HIV).<sup>9,24-26</sup> In addition, great strides have been made in using humanized mice to study the pathogenesis and human-specific therapies in different diseases, such as diabetes, arthritis, graft versus host disease, transplant rejection, and cancer.<sup>25-31</sup> A major breakthrough in the humanization of the mouse immune system came with the discovery that targeted mutation of the IL-2 receptor common gamma chain (IL-2 $\gamma$ ) in NOD/scid mice (termed NOD/scid-IL2 $\gamma^{-/-}$  or NSG mice) greatly enhanced the long-term engraftment of human hemato-lymphoid cells in these severely immuno-deficient mice.<sup>32-35</sup> The objective of this review is to present a brief overview of the recent advances that have been made in the development and use of humanized mice with special emphasis on their use in autoimmune and inflammatory diseases. In addition, we discuss the use of these unique mouse models to define the human-specific immunopathological mechanisms responsible for the induction and perpetuation of chronic gut inflammation.

## THE USE OF IMMUNODEFICIENT MICE AS RECIPIENTS FOR HUMAN HEMATOLYMPHOID CELLS: A LOOK BACK

Adoptive transfer of xenogeneic (human) hematolymphoid cells into healthy wild-type mice results in rapid rejection of these cells and, depending on the type of cells injected, may result in lethal xenogeneic graft versus host disease. To limit these damaging immune responses, immunological tolerance of recipient mice for donor human cells and tolerance of human cells for mouse tissue antigens is required for successful engraftment and survival of human progenitor and/or immune cells.<sup>9</sup> Thus, the ideal hosts for creating this “bidirectional tolerance” are mice devoid of their adaptive and innate immune systems and major histocompatibility complexes (MHCs) I and II. Although these mice have not yet been generated, substantial progress has been made in creating severely immunodeficient mice that support the long-term engraftment of human progenitor and immune cells.<sup>9,25,26,30</sup> These new generation immunodeficient mice are being used as platforms to study the immunological mechanisms associated with hematopoiesis, infectious diseases, cancer, autoimmune diseases, gene therapy, and regenerative medicine.<sup>9,25,26,36,37</sup> In addition, these humanized mice are providing

preclinical models to evaluate the therapeutic efficacy of new drug and vaccine therapies.<sup>9,25,26</sup> Below, we provide a brief history of development of immunodeficient mice and highlight those discoveries that proved to be instrumental for enhancing the long-term engraftment of human hematolymphoid cells.

## Nude Mice

The engraftment of human cells or tissue into immunodeficient mice began more than 50 years ago after the discovery that T-cell-deficient mice failed to reject normal and malignant human tissue.<sup>38</sup> These hairless “nude” (nu/nu) mice were originally observed in a colony of albino mice operated by Dr. N.R. Grist in 1962 at the Virus Laboratory in Ruchill Hospital in Glasgow, Scotland. Dr. Grist subsequently sent the mice to the Institute of Animal Genetics in Edinburgh where Dr. S.P. Flanagan<sup>38</sup> provided a detailed description of the hairless phenotype and described several abnormalities associated with these mice including problems with fertility and neonatal viability, liver disease, and susceptibility to systemic toxoplasmosis. In 1968, Pantelouris reported that nu/nu mice lacked a thymus and thus were devoid of functional T cells<sup>39</sup> (Table 1). A subsequent series of investigations revealed that the nu/nu phenotype arose from a mutation of the *Foxn1* gene previously known as the *whn* gene (winged-helix nude or Hfh11<sup>nu</sup>).<sup>40,41</sup> This pleiotropic transcription factor is

critical for both thymus development and the expression of different hair keratin genes. These T-cell-deficient mice have proven to be one of the most popular mouse models used for engrafting normal or malignant human tissue. Unfortunately, nu/nu mice are of limited use for humanization of the mouse immune system as they do not support engraftment of human hematolymphoid cells.<sup>42</sup> As correctly surmised by Ganick et al,<sup>42</sup> the failure of human immune cells to survive and proliferate in nu/nu mice was due to the presence of functional murine NK cells (see below).

## Severe Combined Immunodeficient and Recombination-Activating Gene-1- or Gene-2-Deficient Mice

The severe combined immunodeficiency (*scid*) mutation was originally described by Bosma et al<sup>43</sup> in C.B.-17 mice. It was ultimately revealed that the *scid* gene encodes for *protein kinase, DNA activated catalytic polypeptide (Prkdc)*.<sup>44,45</sup> Mutation of the *Prkdc* gene prevents the expression of rearranged antigen receptors thereby preventing the development of mature T and B cells<sup>46–51</sup> (Table 1). Because *scid* mice lacked both cell-mediated and humoral immunity, they became the strain of choice for engrafting different populations of human hematolymphoid cells to study hematopoiesis, infectious disease, gene therapy protocols,

**TABLE 1.** Immunodeficient Mice Used for Human Cell and Tissue Engraftment

| Strain                                        | Phenotype                                                                                                                                                                                                        | Limitations                                                                                                                                                                                                             |
|-----------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Nude                                          | Lack T cells                                                                                                                                                                                                     | Intact innate system<br>Fully functional myeloid and NK cells<br>No engraftment of hematolymphoid cells                                                                                                                 |
| SCID                                          | Lack T and B cells                                                                                                                                                                                               | Intact innate immune system<br>Fully functional myeloid and NK cells<br>Functional C5 complement component<br>“Leaky”; T and B cells develop with age<br>Develop thymic lymphoma<br>Very low engraftment of human cells |
| RAG-1 <sup>-/-</sup> and RAG-2 <sup>-/-</sup> | Lack T and B cells                                                                                                                                                                                               | Intact innate immune system<br>Fully functional myeloid and NK cells<br>Very low engraftment of human cells                                                                                                             |
| NOD                                           | Defective innate immune system<br>Abnormal myeloid and NK cell development and function                                                                                                                          | Develop insulin-dependent diabetes mellitus<br>Human cell engraftment has not been studied in the NOD mouse                                                                                                             |
| NOD/SCID                                      | Lack C5 hemolytic complement<br>Lack T and B cells<br>Defective innate immune system<br>Abnormal myeloid and NK cell development and function<br>Lack C5 hemolytic complement<br>Enhanced human cell engraftment | Residual myeloid and NK cell activity innate remains<br>Decreased lifespan; thymic lymphoma                                                                                                                             |

NOD, nonobese diabetic; nude, nu/nu; RAG-1<sup>-/-</sup> and RAG-2<sup>-/-</sup>, recombination activating gene-1 or -2 deficient; SCID, severe combined immunodeficient.

and tumor cell trafficking (reviewed in Ref. 52). Several variations of the human-*scid* (hu-*scid*) chimeric mouse model were developed that used different routes of administration of human cells (or tissue) into irradiated or un-irradiated *scid* mice.<sup>52</sup> Although exciting new data were generated using the different hu-*scid* models, the numbers of human cells that engrafted and proliferated in these mice were very low (0.5%–5% of total *scid* bone marrow cells).<sup>52</sup> Although young *scid* mice were devoid of both T and B cells, investigators found that T- and B-cell receptors undergo age-dependent rearrangements resulting in the formation of functional T and B cells, a phenomenon called “leakiness.”<sup>53–55</sup> In addition, these immunodeficient mice developed life-shortening thymic lymphomas.<sup>47,53,56</sup> Furthermore, Bosma et al<sup>53</sup> observed an increase in the development of thymic tumors in mice that became leaky as measured by the appearance of serum immunoglobulin (Ig<sup>+</sup>).<sup>53</sup> They observed that the incidence of thymic lymphomas increased from 8% in 3 to 5 month old (Ig<sup>−</sup>) *scid* mice to approximately 32% in age-matched (Ig<sup>+</sup>) *scid* mice. Furthermore, they found that almost 60% of the *scid*(Ig<sup>+</sup>) mice developed these malignant lymphomas at 5 to 9 months of age.

The inability of *scid* mice to support robust engraftment of xenogeneic immune cells was not specific for the *scid* strain or to their age-dependent propensity to generate functional T and B cells as human cell engraftment in recombination-activating gene-2-deficient (RAG-2<sup>−/−</sup>) mice was also very low or absent.<sup>52,57</sup> Both the RAG-1 and RAG-2 genes are responsible for the activation of the V(D)J recombination reaction such that deletion of either gene produces mice devoid of T and B lymphocytes<sup>58,59</sup> (Table 1). Unlike *scid* mice, RAG-1<sup>−/−</sup> and RAG-2<sup>−/−</sup> mice (collectively referred to as RAG<sup>−/−</sup> mice) are not “leaky,” do not develop thymic lymphomas and are less sensitive to radiation.<sup>58,59</sup> The reasons for the disappointingly low engraftment of human cells in *scid* or RAG<sup>−/−</sup> mice were found to be due to (1) lack of cross-reactivity between human progenitor cells and essential murine growth factors and (2) the presence of a robust innate immune systems in the lymphopenic recipients. It was clearly demonstrated that human HSCs were incapable of interacting with critical mouse cytokines and growth factors that are required for progenitor cell survival and proliferation.<sup>52</sup> Surprisingly, neither exogenous administration nor transgenic expression of human hematopoietic growth factors (e.g., IL-3, GM-CSF, SCF) increased substantially the engraftment of human cells in *scid* recipients.<sup>52,60</sup> Another confounding limitation that became apparent during this time was that both *scid* and RAG<sup>−/−</sup> mice maintained normal (or increased) numbers of myeloid cells (neutrophils, monocytes, macrophages) and NK cells, as well as normal or increased hemolytic complement; any one of which were capable of destroying xenogeneic progenitor cells.<sup>52,55,60</sup>

### Nonobese Diabetic/*scid* Mice

Recognition of the critical role that innate immune cells (particularly NK cells) played in restricting the engraftment of

human immune cells in *scid* and RAG<sup>−/−</sup> mice prompted investigators to develop new stocks of immunodeficient mice that would allow for greater repopulation of xenogeneic immune cells. A major turning point in these efforts came in 1994 when Shultz et al<sup>55</sup> reported that back crossing nonobese diabetic (NOD) mice onto the *scid* background produced offspring (NOD/*scid* mice) that were devoid of both T and B cells, produced little or no serum immunoglobulin, had low numbers of functional innate immune cells (e.g., NK and myeloid cells) and lacked the hemolytic complement component C5 (Table 1). Unlike their *scid* relatives, <10% of the NOD/*scid* mice developed functional T and B cells at 200 days of age.<sup>55</sup> The rationale for generating NOD/*scid* mice was based on previous studies that were investigating the immunopathological mechanisms responsible for the spontaneous development of insulin-dependent diabetes mellitus in NOD mice.<sup>61</sup> These earlier studies demonstrated that while NOD mice possessed autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells, these animals displayed defective innate immune function including reduced numbers and function of NK and myeloid cells, defective dendritic cell and macrophage function, and the absence of the hemolytic complement component C5<sup>52,62–64</sup> (Table 1). In addition, it was found that NOD mouse-derived macrophages expressed the sigma regulatory protein alpha (SIRPα) receptor that was able to bind to CD47 expressed on human immune cells.<sup>65,66</sup> This receptor–ligand interaction induces an inhibitory or “do not eat me” signal within murine macrophages thereby preventing these phagocytes from engulfing and destroying human progenitor or immune cells.<sup>9,65,66</sup> Thus, the progeny of the NOD-*scid* cross were not only lymphopenic but they also possessed phagocytic tolerance and a defective innate immune system making them more likely to support long-term engraftment of human immune cells<sup>55,67</sup> (Table 1).

The generation of the NOD/*scid* stock initiated one of the most active periods of investigations in the humanization of the mouse immune system. Indeed, for more than 10 years, the NOD/*scid* mouse was considered the “gold standard” for long-term repopulation of human hematolymphoid cells.<sup>25,26,55,68</sup> Investigators found that the engraftment of human PBMCs or HSCs was 5- to 10-fold greater in NOD/*scid* mice when compared with *scid* mice.<sup>25,52,55</sup> During the ensuing decade, it soon became clear that despite its numerous advantages over *scid* or RAG<sup>−/−</sup> recipients, the NOD/*scid* platform possessed its own set of shortcomings that limited its overall utility for humanization studies. For example, it was found that NOD/*scid* mice had a relatively short life span (8 months) due to the development of thymic lymphomas in approximately 70% of these mice by 40 weeks of age.<sup>52,55,69</sup> In addition, while significantly reduced, residual NK and myeloid cell activities limited robust engraftment of human immune cells in these mice.<sup>55</sup> These shortcomings made it clear that additional molecular and/or genetic alterations of this promising mouse strain would have to be made to enhance further the engraftment of human hematolymphoid cells.

## Immunodeficient Mice Devoid of Functional IL-2 Receptor Common $\gamma$ Chain

A fundamental breakthrough that greatly enhanced the long-term engraftment of even greater numbers of human hematolymphoid cells came in 2002 with the development of NOD/scid mice that lacked a functional IL-2 receptor common gamma chain (IL-2 $\gamma$ ).<sup>33</sup> As early as 1995, investigators knew that IL-2 $\gamma$  was a critical component of the high-affinity receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21<sup>70,71</sup> (Fig. 1). Because these cytokines are required for T-, B-, and NK-cell development, targeted mutation of IL-2 $\gamma$  resulted in the loss of receptor-ligand signaling thereby creating mice devoid of T, B, and NK cells.<sup>72–74</sup> Over the next few years, several laboratories developed immunodeficient mice devoid of IL-2 $\gamma$  that differed with respect to strain background and the type of IL-2 $\gamma$  mutation<sup>26,28,68</sup> (Table 2). The nomenclature for describing and categorizing the different IL-2 $\gamma$ <sup>−/−</sup> strains may seem a bit confusing for those readers who are not mouse geneticists. Therefore, we have found it helpful to group the most popular IL-2 $\gamma$ <sup>−/−</sup> strains into 3 major categories depending on whether the IL-2 $\gamma$  mutation has been bred onto the NOD/scid, NOD/RAG<sup>−/−</sup> backgrounds (Table 2). For example, the major NOD/scid-IL-2 $\gamma$ <sup>−/−</sup> strains include the NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup> (NSG) mouse and the NOD.

cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Sug</sup> (NOG) mouse. The IL-2 $\gamma$  mutation in NSG mice is a complete null mutation and thus is not expressed whereas NOG mice express a truncated form of the  $\gamma$  chain that can bind cytokines but does not promote cell signaling. Similarly, the NOD/RAG1<sup>−/−</sup>IL-2 $\gamma$ <sup>−/−</sup> mouse (NOD.Cg-Rag1<sup>tm1Mom</sup>Il2rg<sup>tm1Wjl</sup>; or NRG) contains a complete null mutation of IL-2 $\gamma$ . The third major group of IL-2 $\gamma$ <sup>−/−</sup> mice currently being used for humanization studies consists of BALB/c/Rag1<sup>−/−</sup>IL-2 $\gamma$ <sup>−/−</sup> and BALB/c/Rag2<sup>−/−</sup>IL-2 $\gamma$ <sup>−/−</sup> mice (both are called BRG). These mice express a truncated form of IL-2 $\gamma$  similar to that expressed in NOG mice. We refer readers to several recent reviews that describe the generation and unique characteristics of these different immunodeficient mice.<sup>25,26,28,68</sup>

The development of these immunodeficient IL-2 $\gamma$ <sup>−/−</sup> strains was a defining moment in the quest to humanize the mouse immune system as each of these stocks were shown to engraft human HSCs or PBMCs to a much greater extent than had been observed for any of the previous immunodeficient strains.<sup>25,26,28,68</sup> In addition, these new-generation IL-2 $\gamma$ <sup>−/−</sup> mice do not develop thymic lymphomas and live a normal lifespan of 2 years. These novel platform strains have been particularly useful for studies involved in human immunity, hematopoiesis, infectious disease, autoimmune disease, regenerative medicine, and cancer.<sup>9,25,26</sup>

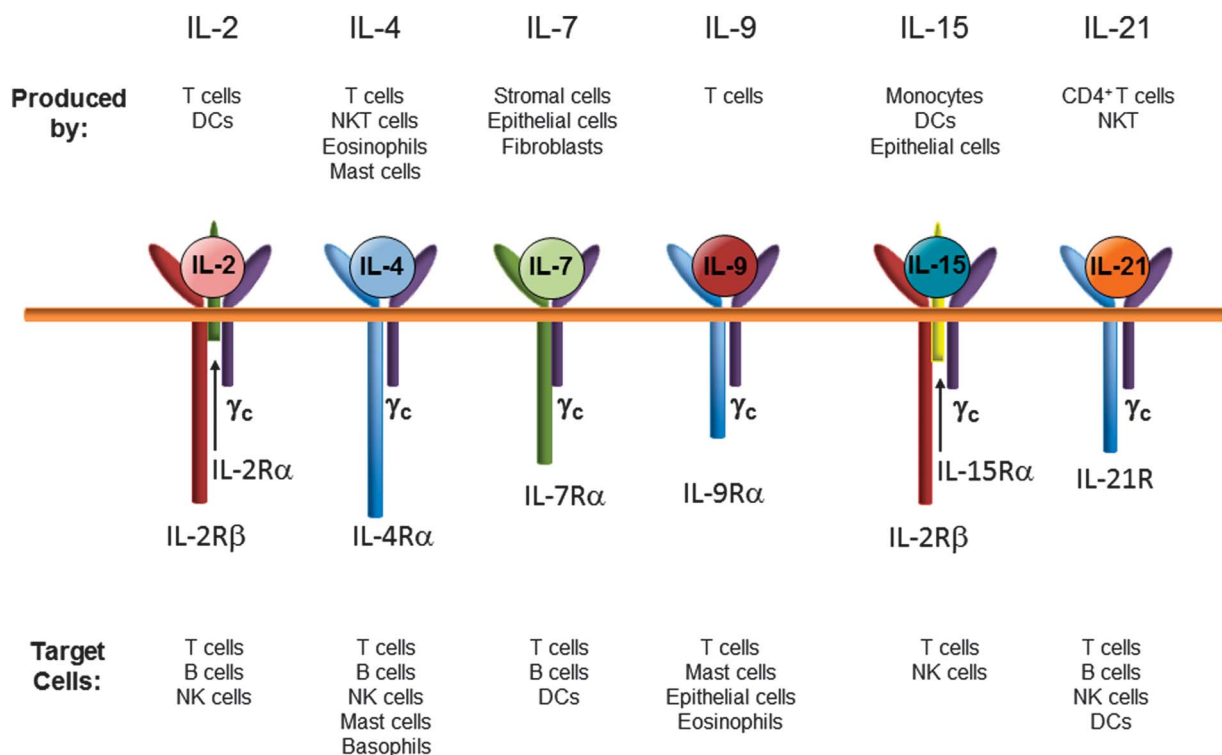


FIGURE 1. The common  $\gamma$  chain plays a critical role in interleukin signaling and the development of immune cells. The common  $\gamma$  chain is associated with receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. These cytokines are produced by different immune stromal cells and epithelial cells. Receptor/ligand signaling is critically important for the generation of different populations of immune cells. Adapted by permission from Macmillan Publishers Ltd: Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. *Nat Rev Immunol*. 2009;9:480–490. Copyright 2009.

**TABLE 2.** Immunodeficient Mice Lacking Functional IL-2 $\gamma$ 

| Group                                                  | Strain                                                              | Name | Characteristics                                                                                                                                                                                                                                                             |
|--------------------------------------------------------|---------------------------------------------------------------------|------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| NOD/scid-IL-2 $\gamma$ <sup>-/-</sup>                  | NOD.Cg- <i>Prkdc</i> <sup>scid</sup> <i>Il2rg</i> <sup>tm1Wjl</sup> | NSG  | The <i>Il2rg</i> mutation is a complete null such that IL-2 $\gamma$ is not expressed<br>The <i>Il2rg</i> mutation encodes a truncated form of the $\gamma$ chain such that it lacks the intracytoplasmic domain; it can bind cytokines but will not promote cell signaling |
|                                                        | NOD.cg- <i>Prkdc</i> <sup>scid</sup> <i>Il2rg</i> <sup>tm1Sug</sup> | NOG  |                                                                                                                                                                                                                                                                             |
| NOD/RAG-1 <sup>-/-</sup> IL-2 $\gamma$ <sup>-/-</sup>  | NOD.Cg-Rag1 <sup>tm1Mom</sup> <i>Il2rg</i> <sup>tm1Wjl</sup>        | NRG  | The <i>Il2rg</i> mutation is a complete null such that IL-2 $\gamma$ is not expressed                                                                                                                                                                                       |
| BALB/c/RAG <sup>-/-</sup> IL-2 $\gamma$ <sup>-/-</sup> | C.Cg-Rag1 <sup>tm1Mom</sup> <i>Il2rg</i> <sup>tm1Wjl</sup>          | BRG  | This strain is generated on a BALB/c background that has the <i>RAG-1</i> <sup>-/-</sup> mutation. The IL-2 $\gamma$ chain lacks the intracytoplasmic domain thereby preventing intracellular signaling                                                                     |
|                                                        | C.Cg-Rag2 <sup>tm1Fwa</sup> <i>Il2rg</i> <sup>tm1Sug</sup>          | BRG  |                                                                                                                                                                                                                                                                             |
|                                                        |                                                                     |      | This strain is generated on a BALB/c background that has the <i>RAG-2</i> <sup>-/-</sup> mutation. The IL-2 $\gamma$ chain lacks the intracytoplasmic domain thereby preventing intracellular signaling                                                                     |

## HUMANIZED MOUSE MODELS

There are currently 3 major humanized mouse models that are based upon the immunodeficient IL-2 $\gamma$ <sup>-/-</sup> strains described above (Table 2). As with any animal model, each of these models has advantages and limitations. These humanized mouse models have been shown to possess different capabilities of engrafting human hematolymphoid cells. For example, investigators have determined that NSG and NRG mice engraft human HSCs and promote T-cell development to a greater extent than do BRG mice,<sup>75</sup> whereas NSG mice support engraftment of larger numbers of human HSCs than do NOG mice.<sup>76</sup> Therefore, the choice of a specific humanized model will depend on the specific questions being posed by the investigator. Theoretically, it should be possible to model most, if not all, of the human autoimmune and chronic inflammatory diseases using one or more of the immunodeficient IL-2 $\gamma$ <sup>-/-</sup> strains. This section describes the generation, uses, and limitations of the 3 major humanized mouse models.

### Engraftment of Human PBMCs into Lymphopenic IL-2 $\gamma$ <sup>-/-</sup> Mice

In 1988, Mosier et al<sup>77</sup> demonstrated that intraperitoneal injection of human peripheral blood leukocytes (PBLs) into *scid* mice (termed Hu-PBL-*scid* mice) resulted in the stable reconstitution of a functional human immune system. Although all populations of circulating leukocytes were injected in the Hu-PBL-*scid* model, the large majority of stably engrafted cells were activated T cells with only very small numbers of human B cells, myeloid cells, or other immune cells present in the *scid* recipients. As discussed in the preceding section, the total number of human T cells that engraft and proliferate in *scid* mice are actually quite low due to the age-related development of mouse T and B cells and the presence of an active innate immune system that together destroy xenogeneic cells.<sup>52,55,60</sup> In addition, *scid* mice developed life-shortening thymic lymphomas thereby limiting the usefulness of this model.<sup>43,53,56</sup> We would like to caution the reader that the use of the term Hu-PBL-*scid* has been used and continues to be used to describe any humanized mouse model

where human PBMCs are injected into lymphopenic recipients, such as a *scid*, NOD/*scid*, or the newer immunodeficient IL-2 $\gamma$ <sup>-/-</sup> strains. To avoid any confusion, we have chosen to focus primarily on the use of lymphopenic IL-2 $\gamma$ <sup>-/-</sup> strains as recipients for human PBMCs in this and the following sections (Table 3). Indeed, the generation of these immunodeficient IL-2 $\gamma$ <sup>-/-</sup> strains greatly advanced the field as investigators observed much greater levels of engraftment of T cells than had been documented in other lymphopenic recipients. In addition, lymphopenic IL-2 $\gamma$ <sup>-/-</sup> strains possessed a much greater lifespan due to the lack of thymic lymphoma development.<sup>25,26,68</sup> Intravenous injection of human PBMCs into irradiated (conditioned) or naive IL-2 $\gamma$ <sup>-/-</sup> strains produces the most robust engraftment of human T cells than in any previous immunodeficient recipient.<sup>78</sup> The *Hu-PBMC-IL-2 $\gamma$ <sup>-/-</sup>* mouse model is currently used to study human immunity, autoimmunity, infectious diseases, regenerative medicine, allograft rejection, and cancer.<sup>9,26,28,68,79</sup> (Table 3). In addition, this model has been used to assess novel cell-based therapies.<sup>36</sup> Although this model is well suited for investigating T-cell-mediated immune responses of patients with immunologic disorders and autoimmune and chronic inflammatory diseases, the “window of opportunity” for performing these types of studies is limited by the development of lethal xenogeneic graft versus host disease (GVHD) within 3 to 4 weeks after T-cell transfer.<sup>80</sup> King et al<sup>80</sup> have demonstrated that xenogeneic GVHD results from the ability of human T cells to recognize and mount aggressive immune responses toward murine MHC class I and II. These investigators have reported a significant delay in xenogenic GVHD when human PBMCs are engrafted into NSG mice devoid of MHC class I and II. Despite this limitation, the Hu-PBMC-IL-2 $\gamma$ <sup>-/-</sup> model has been used successfully to explore immunopathological mechanisms involved in different autoimmune and infectious diseases.

### Engraftment of Human HSCs into Lymphopenic IL-2 $\gamma$ <sup>-/-</sup> Mice

After the early successes of engrafting PBMCs into *scid* recipients, investigators initiated studies to ascertain the ability

**TABLE 3.** Models of Human Hematolymphoid Cell Engraftment into Lymphopenic IL-2 $\gamma$ <sup>-/-</sup> Mice

| Model Name                           | Description                                                                                                                                                                                                                                              | Advantages                                                                                                                                                                                                                              | Applications                                                                                                                                                                                                                                                               | Limitations                                                                                                                                                                                                                                                                                                                                                                                         |
|--------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Hu-PBMC-IL-2 $\gamma$ <sup>-/-</sup> | Lymphopenic IL-2 $\gamma$ <sup>-/-</sup> mice are injected with human peripheral mononuclear cells                                                                                                                                                       | A technically simple model to establish<br>Good engraftment of effector and memory T cells                                                                                                                                              | Induced models of inflammation (e.g. sepsis, arthritis, colitis)<br>Model of xenogeneic GVHD<br>Assess effector functions of T cells obtained from patients with RA, lupus, MS, diabetes, or IBD<br>Human-specific infectious diseases (HIV)<br>Allograft tissue rejection | Only activated/memory T cells are present<br>Lack of mature human B cells, myeloid cells, DCs, platelets, and erythrocytes<br>Xenogeneic GVHD develops after 4–5 weeks due to human T-cell reactivity against mouse MHC molecules<br>Limited primary immune responses                                                                                                                               |
| Hu-HSC-IL-2 $\gamma$ <sup>-/-</sup>  | Lymphopenic IL-2 $\gamma$ <sup>-/-</sup> mice are injected with CD34 <sup>+</sup> HSCs derived from fetal liver, cord blood, bone marrow, or from peripheral blood after G-CSF-mediated mobilization                                                     | Generates a naive human immune system<br>Development of T and B cells, antigen-presenting cells, myeloid cells, and NK cells                                                                                                            | Human hematopoiesis<br>Induced models of inflammation (e.g. sepsis, arthritis, colitis)<br>Engraft human HSCs from patients with autoimmune or chronic inflammatory diseases<br>Human-specific infectious diseases<br>Transplantation biology                              | Low rate of T-cell engraftment<br>Impaired T and B functions<br>No mucosal immune system<br>Lack of expression of human HLA within the thymus prevents education and development of HLA-restricted CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells (delayed type hypersensitivity response is suboptimal)<br>Only small numbers of PMNs, red blood cells, and megakaryocytes are present in the blood |
| BLT-IL-2 $\gamma$ <sup>-/-</sup>     | Lymphopenic IL-2 $\gamma$ <sup>-/-</sup> mice are implanted with small pieces of human fetal liver and autologous thymus under the renal capsule; the mice are then injected with human CD34 <sup>+</sup> HSCs purified from the same fetal liver sample | Human immune system engraftment is much more robust than in the Hu-SRC-NSG model<br>Sustained high level of T cell development; T cells are educated by the human thymus and are HLA restricted<br>Produces human mucosal immune system | Human hematopoiesis<br>Engraft human HSCs from patients with autoimmune or chronic inflammatory diseases<br>Human-specific infectious diseases<br>Transplantation biology                                                                                                  | Surgical expertise and fetal tissue are required<br>Responses to vaccination protocols are limited to IgM antibody production<br>A delayed xenogeneic GVHD (>4 months) occurs in these mice that result from the lack of negative selection against murine antigens in human thymus and/or to lack of peripheral regulation                                                                         |

Hu-PBMC-IL-2 $\gamma$ <sup>-/-</sup>, Human Peripheral Blood Mononuclear Cells engrafted into lymphopenic IL-2 $\gamma$ <sup>-/-</sup> mice; Hu-HSC-IL-2 $\gamma$ <sup>-/-</sup>, Human Hematopoietic Stem Cells engrafted into lymphopenic IL-2 $\gamma$ <sup>-/-</sup> mice; BLT-IL-2 $\gamma$ <sup>-/-</sup>, Bone marrow, Liver, Thymus engrafted into lymphopenic IL-2 $\gamma$ <sup>-/-</sup> mice.

of *scid* mice to support the engraftment and differentiation of human CD34<sup>+</sup> HSCs derived from human fetal tissue or bone marrow.<sup>81,82</sup> Lapidot et al<sup>82</sup> reported that adoptive transfer of human CD34<sup>+</sup> HSCs into *scid* recipients resulted in the generation of small numbers of immature lymphocytes and myeloid cells (Table 3). This model, called the human *scid* repopulating cell *scid* or Hu-SRC-*scid* model, has been used and continues to be used for exploring different immunological aspects of human hematopoiesis, infectious diseases, and adaptive immunity. However, the utility of this model was limited by disappointingly low levels of HSC engraftment and reduced numbers of functional

T and B lymphocytes, myeloid cells, and dendritic cells. The development of immunodeficient IL-2 $\gamma$ <sup>-/-</sup> mice provided a much needed catalyst for developing a more robust humanized immune system in mice.<sup>32–35</sup> A series of studies demonstrated that HSCs derived from fetal liver, umbilical cord blood (UC), bone marrow, and G-CSF mobilized peripheral blood could be used to engraft different lymphopenic IL-2 $\gamma$ <sup>-/-</sup> mice resulting in dramatic increases in engraftment and differentiation of HSCs into different immune cell subsets.<sup>75,83,84</sup> Brehm et al<sup>75</sup> recently compared the engraftment efficiency of CD34<sup>+</sup> HSCs obtained from cord blood in 3 different immunodeficient IL-2 $\gamma$ <sup>-/-</sup> mouse strains at

different ages. They found that engraftment was more robust in irradiated newborns compared with irradiated adults and that T- and B-cell numbers were significantly greater in NSG and NRG mice compared with BRG recipients. Other investigators have found that intrahepatic injection of HSCs also provides an effective mode of delivery of human HSCs for humanizing the immune system.<sup>85,86</sup> Indeed, Misharin et al<sup>86</sup> reported that NSG mice engrafted through intrahepatic injection of human CD34<sup>+</sup> HSCs spontaneously produced human immunoglobulins and generated specific IgG after immunization. In addition to T and B cells, adoptive transfer of human HSCs into IL-2 $\gamma$ <sup>-/-</sup> recipients promotes the formation of small but significant numbers of myeloid cells and DCs.<sup>32–34,87–89</sup>

A major advantage of Hu-HSC-IL-2 $\gamma$ <sup>-/-</sup> models is the absence of xenogeneic GVHD.<sup>82</sup> Because human T cells undergo positive and negative selection within the mouse thymus, these lymphocytes are restricted to mouse MHC and thus, do not react to highly antigenic murine proteins. However, this advantage is also its primary drawback, that is, human T cells are not human leukocyte antigen (HLA) restricted. This means that T cells will not recognize nor react to antigen presented by human antigen-presenting cells that develop within tissue.<sup>90</sup> In addition, HSC engraftment in immunodeficient IL-2 $\gamma$ <sup>-/-</sup> mice have poor T-cell development most likely due to inefficient human thymopoiesis in the mouse thymus.<sup>88</sup> Furthermore, very low numbers of circulating platelets, erythrocytes, and polymorphonuclear neutrophils (PMNs) are produced in following transfer of human HSCs.<sup>26,79,90</sup> Despite these shortcomings, the Hu-HSC-IL-2 $\gamma$ <sup>-/-</sup> models have provided invaluable information on many aspects of human immunobiology.

### Engraftment of Human HSCs into Lymphopenic IL-2 $\gamma$ <sup>-/-</sup> Mice Implanted with Human Fetal Thymic and Liver Tissues

To create a more robust humanized environment that would allow for more complete differentiation of engrafted human HSCs, the bone marrow–liver–thymus (BLT) humanized mouse model was developed (Table 3). In this model, animals are first conditioned using sublethal irradiation and then are implanted with small fragments (~1 mm<sup>3</sup>) of autologous human fetal liver and thymus under the kidney capsules.<sup>9,26,91,92</sup> Recipients of the human tissues are then injected (intravenous) with 1 to 5 × 10<sup>5</sup> autologous human HSCs obtained from the fetal human liver implanted into the recipients.<sup>93</sup> The BLT model, originally developed in NOD/scid mice, convincingly showed long-term engraftment of multilineage human cells, including T and B cells, DCs, monocytes, macrophages, erythrocytes, and platelets.<sup>9,25,91,92,94</sup> In addition, these studies demonstrated that human T cells were HLA restricted and were capable, along with human B cells, of mounting adaptive immune responses *in vivo*.<sup>9,25,91,92,94</sup> Furthermore, BLT mice developed secondary lymphoid tissue (e.g., mesenteric lymph nodes) and human mucosal immune systems thereby making it the model of choice for HIV investigators.<sup>9,25,91,92,94</sup> After the development of immunodeficient

IL-2 $\gamma$ <sup>-/-</sup> mice, investigators compared the relative engraftment efficiencies of human HSCs in the NOD/scid-BLT versus NSG-BLT model with varying results. Although some studies reported similar levels of reconstitution of human HSCs in blood, spleen, liver, and lung in the 2 BLT models,<sup>76,95,96</sup> other studies reported superior human immune cell engraftment in NSG-BLT versus NOD/scid-BLT mice.<sup>32,33,97,98</sup> One important difference between the two BLT models reported by Denton et al<sup>96</sup> was the relative lack of intraepithelial and lamina propria T cells within the intestines of NSG-BLT mice when compared with NOD/scid BLT mice. In a more recent study, Nochi et al<sup>99</sup> demonstrated that cryptopatches and gut-associated lymphoid tissue (GALT) failed to develop in NSG-BLT mice, whereas intestinal T cells and cryptopatches were readily observed in NOD/scid-BLT mice. Furthermore, these investigators demonstrated that cryptopatch development was required for the generation of a functional GALT. Taken together, these two studies highlight the importance of the IL-7/IL-7 receptor signaling for normal GALT development.

Despite model variability, the NOD/scid-BLT and NSG-BLT models are considered two of the best humanized mouse models for studies involved in human hematopoiesis, infectious diseases, acute and chronic diseases, and therapeutic/vaccine development. Despite the fact that BLT mice have a “blended” immune system composed of murine myeloid cells coexisting with a human adaptive immune system, these humanized animals are capable of mounting both direct and indirect delayed type hypersensitivity responses to two different antigenic challenges.<sup>100</sup> Because delayed type hypersensitivity responses are considered the most stringent test for a fully functional and well-integrated immune system, this model is suitable for studies evaluating T-cell–dependent immune responses. As with the other humanized mouse models, the BLT model has a few significant drawbacks including the requirement for small animal surgical skills, the use of human fetal tissue, generation of relatively low levels of mature/functional human granulocytes, myeloid cells, B cells, and a delayed xenogeneic GVHD.<sup>93,101–104</sup> It is not clear why GVHD develops by 4 to 5 months postreconstitution; however a loss of tolerance of human immune cells to murine MHC antigens is considered a distinct possibility. A recent study demonstrates that deletion of either MHC class I or II fails to delay the development of GVHD in the NSG-BLT model.<sup>93</sup> Interestingly, Lavender et al have demonstrated that deletion of CD47 in C57Bl/6-RAG-2<sup>-/-</sup>IL-2 $\gamma$ <sup>-/-</sup> mice renders these triple knock out (TKO; CD47<sup>-/-</sup>RAG-2<sup>-/-</sup>IL-2 $\gamma$ <sup>-/-</sup>) animals resistant to GVHD in the BLT model.<sup>105,106</sup> Based on previous work by Wang et al,<sup>107</sup> the authors hypothesize that during development, phagocytes (i.e., macrophages, DCs) within TKO mice become tolerized to xenogeneic HSCs.<sup>105</sup> Because the expression of SIRP $\alpha$  on macrophages derived from CD47<sup>-/-</sup> mice is very similar, if not identical to that observed on WT macrophages, tolerance observed in TKO-BLT mice is unlikely to be caused by SIRP $\alpha$ -CD47 signaling.<sup>105,107</sup> The major advantages of using this C57Bl/6-based TKO-BLT model versus the NOD/scid-BLT or NSG-BLT model are (1) C57Bl/6-TKO mice

maintain an intact complement system, (2) C57Bl/6-TKO mice are more radiation resistant allowing for more efficient conditioning before human cell engraftment, and (3) genetic manipulations (e.g., gene inactivation; introduction of transgenes) are more easily accomplished because of the ready availability of a variety of different mutant mice on the C57Bl/6 background.<sup>105</sup>

## CURRENT STRATEGIES FOR IMPROVING ENGRAFTMENT OF HUMAN HEMATOLYMPHOID CELLS IN IMMUNODEFICIENT MICE

The advances that have been made in humanizing the immune system of immunodeficient mice over the past 20 years have been truly remarkable. Nevertheless, a number of limitations remain that prevent the development of a fully functional human immune system. For example, virtually all humanized mouse models possess only small numbers of human granulocytes (e.g., PMNs, eosinophils), myeloid cells (e.g., monocytes, macrophages), erythrocytes, and platelets, as well as reduced numbers of fully functional T and B cells.<sup>9,28,79</sup> Many of these deficiencies are most likely due to the lack of cross-reactivity of certain murine cytokines and/or growth factors with human progenitor cell receptors that are known to be important for hematopoiesis and immune system development.<sup>9</sup> However, poor development of peripheral lymph nodes, GALT, and mucosal immune systems undoubtedly contributes to an underdeveloped immune system.<sup>28,79,96,99</sup> The following section discusses some of the new approaches that are currently being used to improve engraftment and differentiation of human HSCs in immunodeficient mice.

### Strategies for Enhancing the Human Innate Immune System

The development, numbers, and function of human PMNs, monocytes, macrophages, and NK cells are generally quite low in the currently used models of humanized mice. For example, human myeloid cells have been found to be less than 5% to 10% of what is found in humans (reviewed in Ref. 9). Although some studies report that these myeloid cells are reasonably functional,<sup>89,108</sup> other studies have described these myeloid cells as phenotypically immature and functionally defective.<sup>109</sup> Furthermore, human NK-cell maturation, function, and homeostasis are also defective in humanized mice owing in part, to NK cell susceptibility to phagocytosis by murine macrophages<sup>97</sup> (discussed below) and lack of transpresentation of hIL15-hIL15R $\alpha$ .<sup>110</sup> Many of the cytokines and growth factors required for innate immune system development and function are produced by nonhematopoietic cells and thus are of murine origin. A recent review by Rongvaux et al<sup>9</sup> presents an extensive list of these cytokines and factors and highlights the significant differences in sequence homologies between the mouse and human proteins. The lack of effective receptor–ligand interactions between human progenitor cells and mouse-derived cytokines and growth factors would be expected to greatly diminish hematolymphoid cell development in immunodeficient mice.

To improve innate immune cell development and function, several genetic and technological approaches are currently being used to promote and/or enhance expression of crucial human cytokines and growth factors that are known to be important in innate immune system development and function, including (1) use of cDNA constructs expressing human genes that can be controlled by ubiquitous or tissue-specific promoters, (2) use of bacterial artificial chromosomes (BACs) that have regulatory elements for gene expression, and (3) use of knock-in technology to replace mouse genes with specific human genes. The cDNA construct approach can lead to transgene expression at non-physiological levels or without temporal and tissue-specific regulation. BACs often include regulatory elements for control of gene expression facilitating gene expression at physiologic levels and at the appropriate time and location. It should be noted that both the cDNA and BAC approaches result in expression of both the mouse and human gene(s) of interest because the homologous mouse gene is left intact. However, knock-in technology replaces the mouse gene with the equivalent human gene resulting in human gene expression without expression of the mouse protein. Indeed, knock-in technology has been used to increase the numbers of myeloid cells in humanized mice expressing human interleukin 3 (IL3) and granulocyte-macrophage colony-stimulating factor (GM-CSF),<sup>111</sup> thrombopoietin,<sup>112</sup> or macrophage colony-stimulating factor (M-CSF).<sup>113</sup> In a more recent study, Rongvaux et al<sup>114</sup> report the generation of mice expressing human genes for M-CSF, human IL-3, human GM-CSF, and human thrombopoietin that were knocked-in to their corresponding mouse loci in immunodeficient RAG-2<sup>-/-</sup>IL-2 $\gamma$ <sup>-/-</sup> mice (termed MITRG mice). They also generated MITRG mice that also express human SIRP $\alpha$  encoded in a BAC (termed MISTRG). Engraftment of human fetal liver-derived CD34<sup>+</sup> HSCs into the MITRG, MISTRG, RAG-2<sup>-/-</sup>IL-2 $\gamma$ <sup>-/-</sup>, and NSG mice resulted in significantly greater myeloid cells in the blood and BM and nearly 10-fold increases in myeloid cells in the lung, liver, and colon of MITRG and MISTRG mice compared with engrafted RAG-2<sup>-/-</sup>IL-2 $\gamma$ <sup>-/-</sup> and NSG mice.<sup>114</sup> Human peripheral blood myeloid cell populations in of MITRG and MISTRG mice were composed predominantly of monocytes indicating that the mouse environment remained insufficient for the development and/or survival of human PMNs. Human NK cells were also detected in tissues at numbers approximately 10-fold higher in MISTRG engrafted mice compared with NSG engrafted mice. These exciting results were tempered by the poor development and survival of human red blood cells in MITRG and MISTRG mice resulting in progressive anemia that developed due to poor mouse erythropoiesis after irradiation preconditioning. Both the MITRG and MISTRG mice are currently commercially available.

Hydrodynamic delivery (through intravenous injection) of plasmids encoding different human cytokines has also been used in humanized mouse models to transiently increase expression of important cytokines and factors known to be important for hematolymphoid system development and function. Using this

delivery system, Chen et al<sup>115</sup> demonstrated that transient expression of human IL-15 and Flt-3/Flk-2 ligand in NSG mice increased the numbers of human NK cells for more than 30 days after HSC engraftment. These investigators also demonstrated that transient expression of GM-CSF and IL-4, M-CSF or erythropoietin, and IL-3 significantly enhanced the generation of DCs, monocytes/macrophages, or erythrocytes, respectively, in Hu-HSC-NSG mice. In more recent studies, Li et al<sup>108</sup> reported that hydrodynamic delivery of a plasmid encoding for human M-CSF enhanced the generation of mature and functional monocytes and tissue-associated macrophages in Hu-HSC-NSG mice. The use of transgenic technology is another approach that is currently being used to enhance expression of human cytokines and growth factors in immunodeficient mice. For example, Billerbeck et al<sup>116</sup> have reported that transgenic expression of human stem cell factor (SCF), GM-CSF, and IL-3 in NSG mice greatly enhanced the generation of myeloid cells after engraftment with CD34<sup>+</sup> HSCs. Surprisingly, these investigators also observed an increase in the numbers of functional CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) in peripheral tissues of these transgenic mice when compared with humanized NSG animals. Another group of investigators have shown accelerated differentiation of human granulocytes and generation of myeloid cells in NSG mice expressing the human transgene encoding SCF.<sup>117</sup>

## Strategies for Enhancing the Human Adaptive Immune System

The currently used humanized mouse models generate, to varying degrees, all lineages of human immune cells. The Hu-HSC models have been found to possess relatively weak primary and secondary immune responses including lack of immunoglobulin (Ig) class switching, whereas NOD/scid-BLT and NSG-BLT models demonstrate more robust adaptive immune responses that include the generation of both IgM and IgG.<sup>9,26,79,91,92,94</sup> As pointed out above, factors that limit the development of a well-integrated and fully functional adaptive immune system include defective cytokine and growth factor-mediated signaling in human progenitor cells, underdeveloped peripheral and mucosal lymphoid tissues, and susceptibility of human lymphocytes to phagocytosis by mouse macrophages.<sup>9,26</sup> A few studies have demonstrated that humanized mice develop weak antigen-specific T-cell responses.<sup>26,118</sup> Human T cells isolated from the spleens of NSG-BLT mice reveal functional deficiencies characterized by loss of expression of certain costimulatory molecules, such as CD27.<sup>119</sup> This specific deficiency could be partially reversed *in vitro* by addition of human IL-2 and IL-7. However, in general, development, maintenance, and function of human T cells in current humanized mice are suboptimal.<sup>9</sup> A few studies have shown, with varying degrees of success, that the development and maintenance of human T cells may be enhanced using delivery of human recombinant IL-7 or IL-15.<sup>34,120,121</sup> Another factor that is critical for T-cell development and function is the interaction between T-cell receptors (TCRs) and MHC class I and II. Several groups of investigators have recently reported the

generation of transgenic IL-2 $\gamma$ <sup>-/-</sup> mice (e.g., NSG, NRG, NOG) expressing human MHC I or II molecules termed HLA I or II, respectively.<sup>9,28,97,122–128</sup> Transgenic expression of HLA I or II increased the numbers and function of human T cells and enhanced antigen-specific immune responses after viral infection or immunization. The future development of transgenic IL-2 $\gamma$ <sup>-/-</sup> mice expressing both HLA I and II but devoid of both murine MHC class I and II will allow human T cells to develop in a HLA-restricted manner.<sup>28</sup>

In contrast to the relative paucity of functional T cells in Hu-HSC-IL-2 $\gamma$ <sup>-/-</sup> mouse models, human B cell numbers are actually quite reasonable. However, a number of different investigators have reported that B-cell development is incomplete and antigen-specific B-cell responses are significantly reduced in this model compared with human responses.<sup>95,119,129–131</sup> Investigators have observed a skewing of B-cell development toward a more immature phenotype with steady-state levels of human IgG at ~1% of that observed in humans.<sup>95,130,131</sup> Although some investigators have shown that immunization or infection of Hu-HSC or BLT mice may increase production of human IgM and IgG, the results have been variable.<sup>131</sup> For example, IgG formation after immunization of humanized mice with tetanus toxoid or viral antigens has not been consistently observed, whereas immunization of BLT mice with 2,4-dinitrophenyl-hapten-keyhole limpet hemocyanin produced antigen-specific generation of IgG1 and IgG2.<sup>94,131</sup> Because B cells isolated from Hu-HSC-IL-2 $\gamma$ <sup>-/-</sup> mice exhibit a highly diverse immunoglobulin repertoire that is very similar to that found in human B cells, it has been hypothesized that humanized mice have the “genetic potential” to mount antibody responses similar to humans.<sup>131</sup> Studies to enhance human B-cell maturation in humanized mice are currently being explored by different laboratories.

The lack of fully mature B cells in humanized mice can be explained in part by the apparent inability of murine B-lymphocyte stimulating factor (BLyS or BAFF) to interact with human progenitor cells. Administration of human recombinant BLyS to NOD *RAG-2*<sup>-/-</sup> *Prf1*<sup>-/-</sup> mice engrafted with human PBMCs increased both human B- and T-cell engraftment.<sup>132</sup> Treatment with human BLyS for 14 days resulted in 40-fold greater IgM and a 10-fold greater IgG production than controls and mounted 10-fold higher IgM and IgG *de novo* antibody response to pneumococcal thymus-independent antigens. Knock-in of human BLyS into immunodeficient mice should be beneficial to enhance humoral and cell-mediated immune responses in humanized mice in Hu-PBMC-IL-2 $\gamma$ <sup>-/-</sup>, Hu-HSC-IL-2 $\gamma$ <sup>-/-</sup> and BLT mice. Hydrodynamic injection of plasmids encoding for human GM-CSF and IL-4 has been reported to increase production of antigen-specific IgG after immunization in Hu-HSC mice.<sup>133</sup> Another reason for reduced humoral responses in Hu-HSC-IL-2 $\gamma$ <sup>-/-</sup> mice may be due to defective T-cell help since human T cells are selected within the mouse thymus based on murine MHC-II rather than HLA-2 as described above. In support of the importance of HLA-II restricted T cells in humoral responses is a recent study reporting that engraftment

of human HSCs into immuno-deficient IL-2 $\gamma$ <sup>-/-</sup> mice expressing the human HLA-2 (HLA-DR4) transgene results in the generation of IgM, IgG (all 4 subclasses), IgA, and IgE after immunization.<sup>122</sup>

Another important determinant for the development of a fully functional adaptive immune system is the presence of peripheral and mucosal lymphoid tissue. Although immunodeficient mice devoid of functional IL2 $\gamma$  allow for greater engraftment of human hematolymphoid cells, there are few if any peripheral lymph node (PLN) or gut-associated lymphoid tissue (GALT) development in these animals due to impaired IL-7/IL-7R signaling.<sup>9,28,96,99</sup> Absence of this important signaling pathway prevents the generation of lymphoid tissue inducer (LTi) cells that are required for PLN and GALT development. Transgenic overexpression of thymic stromal lymphopoietin (TSLP), a protein that has functional overlapping activity with IL-7, increased LTi numbers and restored PLN development in IL-7<sup>-/-</sup> and RAG-2<sup>-/-</sup>IL-2 $\gamma$ <sup>-/-</sup> mice.<sup>134</sup> Thus, treatment with or overexpression of TSLP may be used to restore PLN and GALT development in humanized NSG mice. Although the effect of this approach on PLN and GALT development in immunodeficient IL2 $\gamma$ <sup>-/-</sup> mice reconstituted with human hematolymphoid cells has not been reported, hTSLP transgenic NSG mice have been developed and reported in abstract form.<sup>135</sup>

An underinvestigated aspect of humanized mouse models is the role that intestinal microbiota play in immune system development. Chung et al<sup>136</sup> elegantly demonstrated that the immune system in germ-free mice fully develops in the presence of mouse but not rat or human microbiota. Germ-free mice colonized with human microbiota had markedly reduced CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the intestine along with fewer proliferating T cells, dendritic cells, and low antimicrobial peptide expression compared with those colonized with mouse microbiota. Colonization of the mice with rat microbiota also failed to fully expand the numbers of intestinal T cells. However, segmented filamentous bacteria partially restored T-cell numbers in the human microbiota-colonized mice. Thus, human-specific microbiota is likely required for healthy human immune system development. Whether microbial host interaction is required between the microbial cells and immune cells or microbial cells and host nonhematopoietic cells or both will need to be determined and humanized mice should be an excellent system to define these interactions.

## Eradication of Residual Murine Innate Immune Cells

Residual mouse innate immune function in immunodeficient IL2 $\gamma$ <sup>-/-</sup> mice continues to limit robust hematolymphoid cell engraftment. Although these mice have a complete absence of NK cells due to the deletion of the IL2 $\gamma$  chain that is necessary for IL15R signaling, it is clear that their absence is not sufficient for full human immune cell engraftment.<sup>9,79</sup> Most human cells (especially lymphocytes and erythrocytes) are particularly susceptible to phagocytosis and destruction by murine macrophages and possibly other phagocytic leukocytes (e.g.,

PMNs). A major component of the innate immune system that prevents phagocytic destruction of its hematolymphoid cells is the interaction between the CD47 ligand and SIRP $\alpha$  receptor. As briefly described previously, the SIRP $\alpha$  receptor is highly expressed on macrophages, dendritic cells, and PMNs, whereas its ligand CD47 is expressed on virtually all other cell types. Interaction of CD47-expressing cells with phagocyte-associated SIRP $\alpha$  initiates antiphagocytosis signaling thereby preventing the inadvertent autodestruction of nonphagocytic cells.<sup>9,65,66</sup> Because murine SIRP $\alpha$  does not recognize human CD47, murine phagocytes readily engulf and destroy human hematolymphoid cells. The NOD strain contains an SIRP $\alpha$  polymorphism making it functionally similar to human protein that partially accounts for the improved engraftment in the NOD/scid and NSG strains.<sup>65,66</sup> Genetically modified strains have been developed with transgenic overexpression of human SIRP $\alpha$ <sup>97</sup> or that express murine CD47 in the human xenograft<sup>137</sup> that have enhanced engraftment and immune system development. As discussed previously, Lavender et al have demonstrated that CD47<sup>-/-</sup>RAG-2<sup>-/-</sup>IL-2 $\gamma$ <sup>-/-</sup>BLT mice are resistant to GVHD.<sup>105,106</sup>

A number of other innovative approaches have been used in an attempt to reduce or eliminate macrophage-mediated destruction of engrafted human cells. Clodronate liposomes have been used to kill macrophages in vivo in NOD/scid or NSG mice receiving human CD34<sup>+</sup> fetal liver cells resulting in enhanced survival of human red cells.<sup>138</sup> However, clodronate also kills human macrophages, which will limit its use in studies of immune function with its long-term use limited by toxicity. Brehm et al are evaluating CD11b or CD11c promoter-driven diphtheria toxin receptor expression as a means to selectively eliminate murine myeloid or dendritic cell populations in NSG mice.<sup>28,79</sup> Murine macrophages and PMNs can also be depleted with antibodies, such as RB6-8C5 (specific for macrophages and PMNs) or 1A8 (specific for PMNs alone).<sup>139,140</sup>

The ability to effectively study innate immune responses in autoimmunity or infectious diseases is complicated by the presence of murine TLR expression and reactive oxygen production. To address this, NSG mice devoid of TLR4, MYD88 cytosolic adapter protein, and type 1 interferon (IFN) receptors are currently being generated<sup>79</sup>; however, this approach may have unintended consequences due to disruption of physiologic homeostatic and inflammatory roles for TLR expression in a variety of host cells including intestinal epithelial cells and myofibroblasts.<sup>141,142</sup> In addition, NSG mice deficient for neutrophil cytosolic factor 1 (Ncf1), a gene that controls macrophage and PMN superoxide production,<sup>140,143</sup> are currently being generated to reduce the cytotoxic activity of murine phagocytic leukocytes.<sup>79</sup>

## USE OF HUMANIZED MICE TO STUDY AUTOIMMUNE AND INFLAMMATORY DISEASES

The National Institutes of Health (NIH) estimates that ~24 million Americans suffer from one of the major autoimmune

diseases, such as type 1 diabetes, lupus, rheumatoid arthritis, multiple sclerosis, or the inflammatory bowel diseases. In contrast, the American Autoimmune Related Diseases Association (AARDA) reports that the actual number may be closer to 50 million individuals because the NIH only tracks and reports only those 24 autoimmune diseases with well-documented etiology studies (<http://www.aarda.org/autoimmune-information/autoimmune-statistics/>). In fact, the AARDA states that there are greater than 100 different autoimmune diseases that contribute to direct health care costs of more than \$100 billion per year. Thus, there is an urgent need to develop new and more potent therapeutic agents to treat these lifelong diseases. The development of the new-generation humanized mouse models has produced a great deal of excitement within the autoimmunity research community as in theory, it should be possible to model many of the major autoimmune and inflammatory diseases. The following section describes some of the more recent studies evaluating the pathogenesis and new drug therapies in humanized mouse models of autoimmune and chronic inflammatory diseases.

### Graft-Versus-Host and Host-Versus-Graft Disease

Allogeneic bone marrow transplantation (BMT) may, in some instances, cure certain types of blood cancers and genetic disorders. Despite the long-term success of BMT, the development of graft versus host disease (GVHD) remains a major cause of morbidity and mortality in these recipients.<sup>68,144,145</sup> Although GVHD is known to result from donor innate and adaptive immune cells responding to recipient antigens (i.e., MHC complexes), donor T cells are thought to be the most important disease-producing effector cells. Two types of GVHD have been described in humans and mice including acute GVHD (aGVHD) and chronic GVHD (cGVHD).<sup>68,144,145</sup> Development of aGVHD in mouse models is characterized by systemic inflammatory tissue injury of the intestine, liver, lungs, and skin, whereas cGVHD involves oral and salivary tissue, intestine, joints, liver, lungs, and connective tissue.<sup>68,144,145</sup> Several of the newer immunodeficient IL-2 $\gamma^{-/-}$  mice have been used to examine the immunopathogenesis of GVHD and evaluate new drug therapies to treat this systemic inflammatory disorder. In general, the Hu-PBMC-IL-2 $\gamma^{-/-}$  mouse models seem to model a Th1/Th17-driven aGVHD.<sup>80,102,146–148</sup> One concern with the use of conventional Hu-PBMC-IL-2 $\gamma^{-/-}$  model is that it is, in actuality, a model of xenogeneic not allogeneic GVHD. Covassin et al<sup>149</sup> recently reported the generation of a novel humanized mouse model of allogeneic GVHD. Using NSG mice devoid of murine MHC class II but expressing the human transgene HLA-DRB1-0401 (called NSG-Ab<sup>o</sup> DR4 mice), these investigators found that injection of human DR4-positive CD4<sup>+</sup> T cells failed to induce GVHD in NSG-Ab<sup>o</sup> DR4 mice, whereas injection of CD4<sup>+</sup> T cells obtained from human DR4-negative donors induced allogeneic GVHD. Although the majority of Hu-PBMC-IL-2 $\gamma^{-/-}$  mice do not develop autoantibody-mediated cGVHD, a few studies have reported cGVHD-like immunopathology (with fibrosis) in the

BLT-IL2 $\gamma^{-/-}$  model.<sup>68,93,101–104,150</sup> These models have been particularly useful in testing a variety of different human-specific therapies, such as anti-TNF antibodies, thymus-derived or induced regulatory T cells (Tregs), or mesenchymal stem cells.<sup>80,151–154</sup> A more recent study demonstrates that administration of an antibody directed against HLA-A\*02:01 to NSG mice injected with PBMCs expressing this human antigen enhanced survival of these recipients.<sup>155</sup> These exciting new data may provide a new approach to treating GVHD in HLA-mismatched BMT.

Transplantation of allogeneic organs or tissues also induces potent immune responses in which the host immune system reacts vigorously to the engrafted tissue creating host versus graft disease, more commonly referred to as allograft rejection.<sup>68,156</sup> Despite decades of immunosuppressive therapy, allograft rejection remains a major hurdle for the long-term survival of the transplanted tissue. The use of humanized mice to study the immunopathology of xenograft or allograft rejection has been driven by the realization that the immune responses to human allografts in these animals differ substantially to those observed in the same species (mouse into mouse) allograft rejection models (reviewed in Ref. 68). However, the inability to transplant entire human organs into the mouse has limited allograft rejection studies to using human blood vessels, skin, or islet cells. It should be noted that although a number of important studies have been performed using Balb/c-*scid* beige (SCID/beige) recipients, relatively few studies have used the newer immunodeficient IL-2 $\gamma^{-/-}$  platforms, such as the NSG, NOG, NRG, or BRG mice (Table 2). When human vascular allograft rejection has been studied in a Hu-PBMC-IL-2 $\gamma^{-/-}$  model, investigators have found that engraftment of human arterial vessels and human PBMCs were significantly better than what occurs in the well-characterized Hu-PMC-*SCID/beige* model.<sup>157–159</sup> The use of Hu-PBMC-IL-2 $\gamma^{-/-}$  models has allowed investigators to evaluate new human-specific cell-based therapies in treating vascular allograft rejection.<sup>158,159</sup> In addition to vascular allograft rejection, investigators have used the newer humanized mouse models to explore the immunopathogenesis of skin allograft rejection. Transplantation of human skin in mice is technically easy and once engrafted, the allograft exhibits viable epidermis and dermis and a functioning vasculature. In addition, skin is a highly immunogenic tissue that elicits vigorous immune responses by the recipient.<sup>156,160</sup> Racki et al<sup>161</sup> have reported an important study in which they used NSG mice to examine human skin allograft rejection in the absence or presence of human PBMCs. Surprisingly, they found that transplantation of human skin into NSG recipients elicited extensive perivascular infiltration of large numbers of mouse Gr1<sup>+</sup> granulocytes that ultimately lead to the allograft injury and rejection. Treatment of NSG-engrafted mice with an antibody specific for Gr1 remarkably reduced the leukocyte infiltration and promoted wound healing and graft remodeling. Using this modification to allow allograft survival, the authors were able to demonstrate human PBMC-mediated allograft rejection after human PBMC administration. These studies have allowed investigators to evaluate the therapeutic efficacy of

human-specific therapies, such as the use of human Tregs.<sup>162,163</sup> The new and exciting work being performed on allogeneic islet cell transplantation is discussed in the following section.

## Type 1 Diabetes

Type 1 diabetes (T1D) is a multifactorial autoimmune disease that arises from a complex interaction among genetics, the immune system, and environment. As with other autoimmune and chronic inflammatory diseases, the incidence and prevalence of T1D has increased dramatically over the past 50 to 60 years especially in “modernized” societies.<sup>164</sup> These data coupled with those demonstrating that concordance rates of <50% in genetically identical twins suggest that environmental factors (e.g., intestinal microbiota) may play an important role in the pathogenesis of T1D in genetically susceptible individuals.<sup>164,165</sup> Numerous animal and human studies suggest that immune-mediated destruction of pancreatic  $\beta$  cells involves both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.<sup>164,165</sup> A variety of different rat and mouse models of T1D have been used over the past several decades to investigate the immunopathogenesis and treatment of this autoimmune disease. Because a T1D-like disease was found to spontaneously develop in NOD mice without the need for addition of toxic chemicals or infectious agents, it became and continues to be the most popular animal model for T1D preclinical studies.<sup>19,61,166</sup> Despite the wealth of information that has been generated related to the immunopathogenesis and treatment of T1D using this mouse model, no therapy has been shown to prevent or cure patients with T1D. The differences between mouse and human immune systems and differences in islet cell composition and function may be major contributors to this lack of bench-to-bedside transition.<sup>19,166</sup> The development of humanized mouse models based on the different immunodeficient IL2 $\gamma^{-/-}$  stocks provides investigators with immunologically more relevant animal models for investigating immuno-pathogenesis and treatment of T1D. King et al developed a chemically induced model of T1D in NSG mice in which mice are rendered hyperglycemic through injection (intraperitoneal) of the  $\beta$  cell toxin streptozotocin (STZ). These investigators found that transplant of human islet cells into STZ-treated NSG mice reduced the hyperglycemia created in these mice.<sup>78,167</sup> Injection of human allogeneic PBMCs into NSG engrafted with islet cells abrogated the protective effect resulting in allograft rejection and return to hyperglycemia. Unfortunately, the use of STZ to induce  $\beta$  cell injury is problematic given that the response to STZ can be quite variable and mouse islets/ $\beta$  cells are known to recover from the chemical injury.<sup>78,156,167</sup> In an interesting variation of this model, Zhao et al<sup>168</sup> demonstrate that adoptive transfer of irradiated splenic mononuclear cells (SMCs) derived from NOD mice with active diabetes into NSG mice before engraftment of human T cells induces a mild insulinitis that acts to trigger severe insulinitis and  $\beta$ -cell destruction after engraftment of human PBMCs derived from T1D patients. These investigators show that human T cells migrate to pancreatic islets through the interaction between chemokine stromal cell-derived factor 1 and chemokine receptor CXCR-4. Severe insulinitis and  $\beta$ -cell destruction

correlated with the infiltration of large numbers of human CD4<sup>+</sup> and CD8<sup>+</sup> T cells into the pancreatic islets. Engraftment of irradiated NOD-derived PBMCs or human T1D PBMCs alone did not induce diabetes in the NSG recipients. Taken together, these data suggest that this approach may provide a more immunologically relevant mouse model to study the role of T cells in the pathogenesis of T1D. However, it must be remembered that the Hu-PBMC-IL-2 $\gamma^{-/-}$  models used to investigate the immunological mechanisms responsible for induction of T1D and islet allograft rejection restrict investigators to studying only the role of human T cells in these processes.

A major step forward in generating mouse models to study islet allograft rejection in mice with a fully humanized immune system has been described by Brehm et al.<sup>169</sup> This model is based on the discovery that mice possessing a spontaneous point mutation in the insulin 2 gene spontaneously develop diabetes by 7 weeks of age.<sup>170,171</sup> Investigators determined that disease was due to insulin 2 protein misfolding resulting in induction of the unfolded protein response followed ultimately by  $\beta$ -cell apoptosis.<sup>172,173</sup> The diabetes/hyperglycemia that develops in these mice (called *Ins2<sup>Akita</sup>* mice) is not associated with obesity or insulinitis. Backcrossing *Ins2<sup>Akita</sup>* mice with NRG mice produced immunodeficient offspring that develop spontaneous hyperglycemia by 3 to 6 weeks of age.<sup>19,156,169</sup> Investigators demonstrated that normoglycemia could be achieved by transplantation of human or mouse islet cells.<sup>169</sup> They also found that within 1 month after engraftment of allogeneic human HSCs into normoglycemic, human islet cell-containing NRG-*Ins2<sup>Akita</sup>* mice, the majority (60%) of mice became hyperglycemic after rejection of allogeneic islet cells.<sup>169</sup> Thus, this modification of the Hu-HSC-IL-2 $\gamma^{-/-}$  model may offer new insights into the treatment and immunopathogenesis of allograft rejection. It should be noted that these data contrast with those reported by Jacobson et al<sup>174</sup> who found that human islet cells were not rejected by BRG mice engrafted with allogeneic HSCs. The reasons for these differences are not readily apparent at the current time. With refinement of these current humanized models and development of new models that more accurately mimic the rejection processes, investigators will be in a position to evaluate novel human-specific therapies to reverse or even prevent allograft rejection. New cell-based therapies that include administration of ex vivo-expanded Tregs or bone marrow-derived mesenchymal stem cells are showing great promise in prolonging islet allograft survival in diabetic mice.<sup>175–177</sup>

As discussed above, T1D is a polygenic disease that seems to be mediated by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.<sup>164,165</sup> Recent genome-wide association studies have confirmed earlier reports demonstrating that the greatest genetic susceptibility to T1D is localized to the class II region of the HLA complex on chromosome 6.<sup>164,165</sup> Indeed, >90% of all pediatric patients with T1D cases possess HLA-DR3,DQB1\*0201 (called DR3-DQ2) or HLA-DR4,DQB1\*0302 (called DR4-DQ8).<sup>164,165</sup> There is good evidence to suggest that priming and activation of CD4<sup>+</sup> T cells is critical to providing help to CD8<sup>+</sup> cytotoxic T cells that ultimately destroy islet  $\beta$  cells (reviewed in Refs. 164 and 165). Activation

of CD4<sup>+</sup> T cells occurs through the interaction between the CD4<sup>+</sup> T-cell receptor (TCR) and autoantigens presented by the HLA-DR or HLA-DQ complexes residing on antigen-presenting cells in the draining lymph nodes and/or within the pancreatic islets. Examples of known T1D-related autoantigens include preproinsulin (PPI), insulinoma-associated antigen-2, glutamic acid decarboxylase, zinc transporter, and islet-specific glucose-6-phosphatase catalytic subunit (IGRP) to name just a few.<sup>164,178</sup>

The generation of transgenic IL-2 $\gamma$ <sup>-/-</sup> mice expressing HLA class I or II has provided investigators the tools to evaluate the roles of different T-cell subsets in autoantigen-driven insulinitis and diabetes in vivo. Recent work by Viehmann Milam et al<sup>178</sup> shows that engraftment of HLA-matched human PBMCs (DRB1\*0401) that were pulsed (ex vivo) with autoantigen-derived peptides (PPI or IGRP) into NSG-DR *tg* mice induced the infiltration of T cells into mouse islets. Although T-cell infiltration was observed after transfer of healthy autoantigen-pulsed PBMCs, insulinitis was found to be more severe after engraftment of autoantigen-pulsed PBMCs obtained from diabetic donors. Furthermore, the authors observed decreased insulin staining and  $\beta$ -cell injury in NSG-DR *tg* mice engrafted with diabetic versus healthy PBMCs. Although overt diabetes was not observed in this model, this is a very good mouse model for investigating the mechanisms responsible for the selective trafficking of autoreactive T cells to pancreatic islets and the mechanisms by which they damage the tissue. Other investigators have used a similar approach to ascertain the role that CD8<sup>+</sup> T cells play in the induction of insulinitis in the humanized IL-2 $\gamma$ <sup>-/-</sup> mouse models. Whitfield-Larry et al<sup>179</sup> demonstrated that transfer of HLA-A2-matched PBMCs from patients with T1D but not from healthy donors into NSG-HLA-A2 transgenic mice induced selective and robust islet inflammation. The inflammatory infiltrate was composed of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Importantly, the authors showed that invading CD8<sup>+</sup> T cells were autoantigen-specific that could be stimulated to produce large amounts of interferon- $\gamma$  after ex vivo stimulation with different T1D-associated autoantigens. Similar to the model described above, these mice did not develop overt diabetes. Nevertheless, this model represents another important step forward that will be useful for defining the cellular and immunological mechanisms responsible for islet-specific lymphocyte trafficking and HLA-restricted antigen responses. In a third study, Unger et al<sup>180</sup> reported that injection of a IGRP-specific CD8<sup>+</sup> T-cell clone generated from diabetic donor blood into NSG-HLA-A2 *tg* mice induced severe insulinitis and islet cell damage. These data clearly showed that autoreactive CD8<sup>+</sup> T-cell clones are capable of trafficking specifically to pancreatic islets where they mediated injury to HLA-expressing beta-cells. The fact that none of the 3 NSG-HLA transgenic mouse models described above develop hyperglycemia/overt diabetes suggest that engraftment with both CD4<sup>+</sup> and CD8<sup>+</sup> T cells may be required for full expression of disease. Transgenic expression of both HLA class I and II molecules may help to promote development of diabetes provided that disease can develop within 4 to 5 weeks, that is, before the onset of GVHD. Furthermore, the use of HLA *tg*

versions of the Hu-HSC-NSG or BLT models may provide the necessary innate and adaptive immune cells required for full expression of disease in the absence of GVHD.

## Multiple Sclerosis

MS is an autoimmune disease characterized by chronic inflammation and demyelination of the central nervous system.<sup>181</sup> Although the precise etiology of MS remains to be defined, there is accumulating evidence that the inflammation and consequent neuronal demyelination occurs from aberrant T-cell responses to myelin-derived antigens, such as proteolipid protein (PLP) and myelin basic protein (MBP).<sup>181–183</sup> The strongest genetic factor associated with the development of MS is expression of certain HLA class II molecules, such as the HLA-DRB1\*1501 allele, which confers a ~3-fold increase in risk.<sup>184</sup> A large number of studies, using different mouse models of MS have been used over the past 30 years that have greatly advanced our understanding of the immunopathological mechanisms responsible for CNS inflammation and injury.<sup>181</sup> The most popular mouse models have included induced models based on autoimmune encephalomyelitis (EAE) studies, spontaneous models of MS and more recently, transgenic mice expressing different human HLA alleles.<sup>181</sup> Although these models have been instrumental in revealing important cellular and molecular pathways responsible for induction of CNS inflammation, they did not recapitulate human MS. An obvious reason for the differences is that CNS inflammation in mice is mediated by “mouse-specific” effector cell mechanisms. More recently, investigators have used the Hu-PBL-NSG model to produce a subclinical autoimmune model of encephalomyelitis.<sup>185</sup> For this model, investigators cotransferred myelin oligodendrocyte glycoprotein (MOG)-pulsed human DCs together with healthy human PBMCs obtained from the same donor as DCs into NSG mice. Control mice were injected with both human PBMCs and DCs that were not pulsed with antigen or pulsed with irrelevant peptides. After the initial injections, the humanized mice received a “booster” injection (subcutaneous) of immature/nonpulsed DCs followed 12 hours later with a subcutaneous injection of MOG (or irrelevant peptide) suspended in Freund’s complete adjuvant (FCA) to induce DC maturation. The authors found that at 24 days after engraftment of MOG-pulsed PBMCs, significant CNS inflammation could be observed. The CD45<sup>+</sup> inflammatory infiltrate was composed primarily of human CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Although human T cells obtained from spleens of humanized NSG mice were shown to mount robust MOG-specific immune responses, the classic signs of EAE (e.g., paralysis and paresis) were not observed in this novel model.<sup>185</sup> Nevertheless, this new model of myelin-induced CNS inflammation will be a valuable tool to assess new drug therapies targeted at T-cell trafficking and inflammatory cytokine production in a humanized system in vivo.

## Autoimmune and Inflammatory Rheumatic Diseases

Autoimmune and inflammatory rheumatic diseases represent the largest subset of autoimmune diseases affecting over

45 million individuals in the United States.<sup>186–188</sup> These chronic inflammatory diseases are characterized by painful swelling of the joints, muscle, and/or tissue. The most prevalent of the rheumatic diseases include rheumatoid arthritis (juvenile and adult), systemic lupus erythematosus, Sjogren's syndrome, the spondylarthritides (e.g., ankylosing spondylitis, reactive arthritis, psoriatic arthritis, enteropathic arthritis), and systemic sclerosis.<sup>188</sup> Although a number of different mouse models have been developed to act as surrogates for some of the major rheumatic diseases, the use of humanized mice to study human-specific immune mechanisms and treatment modalities in these disease models have only recently been attempted. Below, we describe the use of immunodeficient IL-2 $\gamma$ <sup>-/-</sup> stocks to generate mouse models of some of the major rheumatic diseases.

### Rheumatoid Arthritis

A variety of mouse models have been used to study various aspects of induced or spontaneous arthritis; however, none of these models completely recapitulate the immunopathogenesis of human rheumatoid arthritis (RA).<sup>189</sup> Similar to diabetes, the susceptibility to RA is associated with certain HLA class II alleles. Previous studies have demonstrated that immunization of transgenic mice expressing the human HLA-DR4 or HLA-DR1 allele with type II collagen (CII) induces chronic joint inflammation. As with other mouse models of autoimmunity, interpretation of data generated using these *tg* models is limited by the fact that joint inflammation is driven entirely by the mouse immune system.<sup>190,191</sup> Recent studies have attempted to use the new-generation humanized mouse models to examine arthritis pathogenesis and treatment. Based on intriguing clinical observations demonstrating that Epstein-Barr virus (EBV) infection is associated with the development of RA in humans,<sup>192,193</sup> Kuwana et al<sup>194</sup> have developed an interesting model of erosive arthritis based on the Hu-HSC-NOG mouse model. These investigators observed that 65% of the NOG mice that were engrafted with human HSCs and then infected with EBV exhibited extensive arthritis in knee and ankle joints beginning at 26 days postinfection. No joint inflammation was observed in the absence of EBV infection. Histopathological analysis revealed remarkable synovial proliferation and extensive bone destruction and edema all of which appear very similar to those observed in human RA. In addition, they found that the inflamed joint and synovial tissue contained large numbers of human CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B cells, and macrophages. These interesting results demonstrate that EBV infection is capable of inducing human immune cell-mediated erosive arthritis that is quite similar to human RA. In another study, Misharin et al<sup>186</sup> report the development of a humanized mouse model of acute arthritis. These investigators used a similar humanized mouse model in which NSG mice were engrafted with human CD34<sup>+</sup> HSCs and then subjected to intra-articular injection of FCA 16 weeks after HSC engraftment to induce acute arthritis. At 7 days after FCA injection, mice developed histological evidence of arthritis that was characterized by erythema, edema, pannus formation, and infiltration of human

lymphocytes, as well as human and mouse PMNs and macrophages. Furthermore, these investigators demonstrated that FCA-induced arthritis could be attenuated by administration of the TNF inhibitor etanercept.

### Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a multiorgan autoimmune disease that affects primarily the joints, kidneys, and skin but may also involve the lungs and central nervous system. This rheumatic disease is characterized by production of various autoantibodies and deposition of immune complexes into the different tissues. Although investigators believe that one or more of the autoantibodies generated during the development of disease is/are important in disease pathogenesis, the precise etiology of SLE has yet to be defined. As with RA and other autoimmune diseases, induced and spontaneous mouse models of SLE have greatly advanced our understanding of those immune cells and mediators that promote disease; however, development of disease in many of the mice is strain specific and does not recapitulate certain immunopathological aspects of human SLE.<sup>195,196</sup> In an attempt to model SLE in mice with a partially humanized immune system, Andrade et al<sup>195</sup> have used the Hu-PBMC-BRG model to create mice that develop most or all of the pathological hallmarks observed in human SLE. These investigators found that human PBMCs obtained from either SLE or healthy donors (HD) engrafted very well in NRG recipients at 3 to 4 weeks after transfer. In addition, the circulating numbers of CD4<sup>+</sup> T cells was remarkably reduced in SLE- versus HD-engrafted mice, whereas CD8<sup>+</sup> levels were substantially greater in SLE versus HD-engrafted animals. The authors found no significant differences in human IgG and anti-dsDNA antibody levels in SLE versus HD-engrafted mice. However, if mice were injected with PBMCs obtained from a SLE donor that was expressing high levels of anticardiolipin antibodies (aCL), all engrafted mice expressed 2 to 3 times more aCL than did the other SLE or HD-engrafted mice. At 4 to 8 weeks after engraftment, SLE-NRG mice were found to develop proteinuria and human IgG immune complex deposition within the kidney glomeruli. Microthrombi and CD8<sup>+</sup> T-cell and B-cell infiltration were observed in NRG mice engrafted with PBMCs obtained from SLE donors that expressed high titers of aCL. Taken together, this novel model seems to recapitulate some of the pathological features observed in human SLE.

### Sjogren's Syndrome

Sjogren's syndrome (SS) is one of the most prevalent autoimmune diseases that affect ~3% of the population. Individuals who suffer from SS experience systemic inflammation however disease is usually localized to salivary and lacrimal glands.<sup>197</sup> Histopathological analyses reveal the infiltration of large numbers of CD4<sup>+</sup> T cells and the upregulation of different proinflammatory cytokines.<sup>197</sup> Because the pathology observed in these mouse models of SS does not reproduce some of the key alterations described in human disease, investigators are just beginning to explore the use of humanized mice to study the

immunopathological mechanisms responsible for induction of this prevalent disease. In the first and only study reporting the use of humanized mice in SS investigations, Andrade et al showed that adoptive transfer PBMCs obtained from SS donors into NSG mice resulted in increased serum levels of a variety of human inflammatory cytokines, including IFN- $\gamma$ , IL-17, IL-6, and TNF $\alpha$  when compared with NSG engrafted with healthy donor (HD) PBMCs.<sup>198</sup> In addition, these investigators observed the infiltration of significantly larger numbers of human CD4<sup>+</sup> T cells into the salivary and lacrimal glands of the SS- versus HD-engrafted NSG mice. Although significantly larger numbers of human CD8<sup>+</sup> T cells and B cells were observed in both glands in SS- versus HD-engrafted animals, the absolute numbers of these lymphocytes were much smaller than those observed for CD4<sup>+</sup> T cells.<sup>198</sup> This inflammatory infiltrate correlated well with loss of target organ function as saliva production was significantly decreased by 36% at 4 weeks after transfer of SS-derived PBMCs. This exciting new model will likely provide investigators the unique opportunities to not only explore T-cell-dependent mechanisms of tissue injury but will also provide an important platform to assess new therapeutic agents to treat this rheumatic disease.

## Inflammatory Bowel Diseases

Crohn's disease (CD) and ulcerative colitis (UC) are two of the best-characterized inflammatory bowel diseases. They are chronic and unrelenting inflammatory disorders of the small bowel and/or colon that affect approximately 1.5 million people in the United States with a calculated annual cost for both medical expenses and work loss of almost \$4 billion.<sup>199</sup> Although the etiologies of CD and UC have yet to be fully elucidated, there is growing experimental and clinical evidence to suggest that chronic gut inflammation results from a dysregulated immune response to components of the normal gut flora in genetically susceptible individuals. This paradigm is based on both animal and human studies demonstrating: a) intestinal bacteria are required for the development of chronic gut inflammation in genetically susceptible mice, b) increased numbers of bacteria associated with epithelial cells and the lamina propria of patients with IBD, c) certain subsets of patients with IBD express polymorphisms in genes that are involved in intracellular processing and killing of bacteria, and d) IBD is associated with major alterations in the luminal composition of the microbiota, a situation termed dysbiosis. Whether dysbiosis is a cause or consequence of IBD has yet to be determined. Neither CD nor UC are considered classical autoimmune diseases since no convincing data have been reported demonstrating that these diseases develop from autoaggressive immune responses to intestinal tissue self-antigens. If however, we consider the trillions of intestinal microbes as a "virtual organ" that is present for our entire lifetime, we may wish to redefine our microbiota as "self."<sup>200,201</sup> Using this definition of self, it is quite reasonable to consider IBD as just another type of autoimmune disease. Few autoimmune or chronic inflammatory diseases have garnered more attention from their respective research communities in such a short period of

time as has IBD. In just the last 20 years, thousands of studies have been published using mouse models of IBD to define disease pathogenesis and potential treatment modalities. However, the success of translating the hundreds of promising preclinical studies into effective new therapies to treat IBD has been unimpressive. The reasons for the lack of bench-to-bedside transition have already been described in previous sections and are beyond the scope of this overview. We do however refer the reader to recent reviews that examine this subject.<sup>1,8</sup>

In view of the large number of preclinical studies that have been and continue to be performed, it is rather surprising to find that very few studies have attempted to model IBD in humanized mice. Nolte et al<sup>202</sup> recently described a model of chemically induced IBD using the Hu-PBMCs-NSG mouse model. These investigators engrafted NSG mice with human PBMCs obtained from healthy donors (HD) or from individuals suffering from UC or atopic dermatitis (AD). At 7 days after transfer, mice were sensitized, by dermal application, to the vehicle (ethanol) or the hapten oxazalone (OXA). The mice were then challenged 24 hours later through intrarectal administration of either 50% ethanol or OXA in 50% ethanol.<sup>202,203</sup> Histopathological analyses of the different groups revealed that mice challenged with ethanol or OXA developed the same degree of colonic inflammation that consisted of edema, fibrosis, crypt dropout, and infiltration of T cells into the lamina propria. No significant differences were observed in the histopathology scores among NSG mice engrafted with HD, UC, or AD PBMCs and challenged with OXA. Although this study presents interesting data related to "haptens"-induced colitis in humanized mice, it illustrates a major problem associated with mouse models of chemically induced colitis: the use of erosive/toxic chemicals such as ethanol creates severe inflammation that may mask more subtle alterations induced in humanized mice. Another limitation with the use of this type of chemical model is that it lacks participation by the human innate system that will likely be important to faithfully model human IBD.

Using a similar approach as described for the OXA model, Goettel et al<sup>204</sup> recently reported in abstract form only the use of transgenic NSG mice that expressed human HLADR1 but lacked the murine MHC class II (called NSGAb0DR1 mice) as a platform for inducing acute colitis. These investigators engrafted the NSGAb0DR1 mice with HLA-matched CD4<sup>+</sup> T cells and then sensitized (through dermal administration) the mice with the hapten, trinitrobenzene sulfonic acid (TNBS). One week later, these mice received an intrarectal challenge with TNBS in ethanol resulting in clinical and histologic evidence of intestinal inflammation that was not observed in the NSGAb0DR1 mice receiving TNBS in ethanol but no human T cells. Although promising, the TNBS model suffers from many of the same limitations as do the other chemically induced models of gut inflammation that use erosive/toxic substances to "break" the mucosal barrier.<sup>1</sup> A second preliminary study reported by this same group (abstract form only) showed that engraftment of NSGAb0DR1 mice with HLA-matched CD34<sup>+</sup> HSCs from

a patient with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome induced systemic and lethal autoimmunity similar to that observed in humans with IPEX and mice that lack functional Foxp3 transcription factor.<sup>205</sup> It will be of great interest to see how this type of humanized mouse model may be used in the future to model human IBD in a well-controlled and statistically powered study.

A novel mouse model of allergen-induced intestinal inflammation has recently been developed by Weigmann et al.<sup>206</sup> These investigators engrafted PBMCs from donors with clinical allergy to grass, birch pollen, or hazelnut together with their respective allergen (intraperitoneal) into NSG mice. At 21 days after engraftment, mice were challenged orally or rectally with their specific allergen. Mice engrafted with allergic PBMCs developed colonic inflammation that consisted primarily of human lymphocytes and neutrophils in both the oral or rectal challenged groups but not following saline challenge. Mice engrafted with nonallergic (healthy) PBMCs did not develop colitis when challenged with the allergens.<sup>206</sup> Furthermore, the colitis could be significantly attenuated by administration of anti-human IgE antibody but not with control human IgG. Although it was not clear whether the PMNs infiltrating the colon were of human and/or murine origin, this study clearly demonstrates that human leukocyte trafficking to the intestine is largely intact and sufficient to develop colonic inflammation. Whether trafficking is sufficient to allow the full spectrum of IBD pathology remains to be determined.

## LOOKING FORWARD

The development of humanized mouse models of IBD offers investigators the ability to study the pathophysiology of CD or UC in a manner not possible in humans. Preclinical therapies can be tested, and the environmental influences such as diet and the gut microbiome can be tested and defined. However, a number of issues and fundamental questions remain regarding our ability to generate these humanized mouse models. It may be a bit naive to believe that chronic gut inflammation can be generated by simply engrafting HSCs obtained from patients with CD or UC into any of the immunodeficient IL-2 $\gamma^{-/-}$  mice. Because genetically identical twins express relatively low concordance rates for CD (~30%–35%) or UC (~15%–20%), environmental factors (e.g., intestinal bacteria, diet, housing) most likely will play important roles in model development.<sup>207–212</sup> Although mouse and human flora are in general, fairly similar in composition, significant differences do exist.<sup>9</sup> Indeed, the enormous impact that mouse versus human microbiota has on murine intestinal immune system development<sup>136</sup> suggests that the antigenic stimuli may require colonization with human flora, disease specific flora, or autologous flora specific to the PBMC or HSC donors. If donor specific flora is required for induction of disease, antibiotic treatment followed by human fecal transfer may be sufficient and alleviate the need for a gnotobiotic facility.<sup>213</sup> Transfer of autologous PBMCs into immunodeficient

mice engrafted with HSCs from patients with IBD may also prove useful in providing reactive T cells with immunologic memory to induce disease. Further refinements could include treatment with indomethacin or piroxicam to initiate and synchronize the onset of disease, an approach that has been used in the IL10 $^{-/-}$  model of chronic colitis.<sup>214,215</sup> Another concern relates to the extent of engraftment of CD34 $^{+}$ -derived endothelial cells in mice.<sup>9</sup> Although the incomplete engraftment of human endothelial cells may influence trafficking of human leukocytes to lymphoid and nonlymphoid tissue, current evidence suggests that leukocyte infiltration and intestinal inflammation do occur in the xenogeneic GVHD that develops in the Hu-PBMC-IL-2 $\gamma^{-/-}$  and BLT-IL-2 $\gamma^{-/-}$  mouse models.<sup>103,154</sup> In summary, the outlook for the generation of even better humanized mouse models to study autoimmune and chronic inflammatory diseases is very bright. It will be very interesting to following the evolution of this exciting aspect of animal model development over the next few years.

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## ERRATUM

### Determinants of Intestinal Permeability in Healthy First-degree Relatives of Individuals with Crohn's Disease: ERRATUM

In the article on page 879, volume 21, issue 4, there is an error in the text of the abstract. The first sentence of the abstract should appear as follows: "Results: One thousand, one hundred ninety-six white FDRs were included."

## REFERENCE

- Kevans D, Turpin W, Madsen K, et al. Determinants of Intestinal Permeability in Healthy First-degree Relatives of Individuals with Crohn's Disease. *Inflamm Bowel Dis*. 2015;21:879–887.