2014

Comparisons between the NOD and NOR mouse: insight into diabetes pathogenesis and protection

Joseph Guy Daft
University of Alabama at Birmingham

COMPARISON OF THE NOD AND NOR MOUSE: INSIGHT INTO DIABETES PATHOGENESIS AND PROTECTION

by

JOSEPH G. DAFT

ROBIN G. LORENZ, COMMITTEE CHAIR
DANIEL F. BALKOVETZ
CHARLES O. ELSON III
JOHN F. KEARNEY
MOON H. NAHM
HUBERT M. TSE

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2014

COMPARISON OF THE NOD AND NOR MOUSE GIVES INSIGHT INTO PROTECTION FROM TYPE 1 DIABETES PATHOGENESIS

JOSEPH G. DAFT

MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

Type 1 Diabetes (T1D) is defined as the selective immune destruction of insulin producing beta cells within the islet. A new emphasis has been put on the role of the gastrointestinal (GI) tract in T1D; however, there is much more to learn about this relationship. Distinct differences have been observed in the intestinal permeability, barrier function, commensal microbiota, and mucosal innate and adaptive immunity of patients and animals with T1D, when compared to healthy controls. The non-obese diabetic (NOD) mouse and the BioBreeding diabetes prone (BBdp) rat are commonly used to models to study T1D in humans. Most murine studies use the common BALB/c or C57BL/6 as negative controls for T1D studies, which may not be the most relevant control animals.

To investigate T1D using the NOD model we used the non-obese diabetic resistant (NOR) mouse as a control model. NOR mice are approximately 80% genetically identical to NOD mice and importantly like NOD mice we have observed that NOR also have a permeable intestine. Comparison between these two models has allowed us to begin understanding why NOR mice are protected from T1D despite many similarities to NOD mice.

While comparing NOD and NOR mice we observed a difference in microbiota between the two strains. NOD mice have a higher frequency of Bacteroidetes and a lower frequency of Firmicutes when compared to NOR mice, which is supported by literature

when comparing healthy subjects and subjects with T1D. To determine what lead to differing microbiota between the two strains we investigated the role of antimicrobial peptides (AMPs) and mucins in regulation of the microbiota. We observed decreased AMP and mucin expression (mRNA) in the absence of bacteria in NOD mice compared to NOR mice. Importantly a mucus kill assay using *E. coli* provided functional evidence of a decreased ability of NOD mice to regulate bacteria when compared to NOR mice.

Keywords: Type 1 diabetes, microbiota, antimicrobial peptides, mucins, cross-fostering

ACKNOWLEDGMENTS

This all started when Robin took me into her lab after my previous mentor left for another institution. Thank for the incredible opportunity to join your lab and be mentored by you. You have been an incredible boss both in and out of science. Your work ethic is inspiring and impossible to keep up with! Your support of my life outside of science is something I will never forget and has given me a model to use when I mentor students in my coming years.

To my brother Paul, you have been there from start to finish. Having a brother in the same program is an experience that not many will ever have. You have been my best friend since April 11, 1987, and going through the PhD program with you has certainly been interesting. Sharing the frustrations of science with you when mom and dad just didn't understand was huge. I wish you all the best and am stoked for where science will take you.

Thank you to members of the Lorenz lab past and present: Scott, Liz, Jamie, Mason, Kyle, Tay Tay, Dr. Martin, Bree, and Zollie. Things have certainly never been boring and the beer and food have been great, we even managed to get a little science done too.

To the Beer Friday crew: Paul, Rob, Juan, Scott, Melson, Rachel, Ben, and Kim. No matter how bad the week was, we could always kick back and relax at Dave's. And of course I haven't forgotten Randy. Not only were you a great administrator, but you were also an incredibly supportive friend who was always there for me.

To my South Highland family thank you for your prayers and guidance as I've moved through life in Alabama. Thank you to my "youth," I had such a great time hanging out with you and ministering to you. A special thanks to Jim, Doug, and Rachel, you are all incredible examples of what it means to live a Christ like life and I look forward to following in your footsteps.

To my parents, thank you for your support from start to finish. You've had confidence in me when I didn't. I always took great comfort in knowing that no matter how science was going you loved and supported me anyway.

Last, but not least, my wife Sheila. I cannot imagine finishing up without you by my side. You have been such a blessing and I thank God for bringing you into my life. Good and bad, I look forward to having you with me, I love you.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figures *Page*

INTRODUCTION

CROSS-FOSTERING IMMEDIATELY AFTER BIRTH INDUCES A PERMANENT MICROBIOTA SHIFT THAT IS SHAPED BY THE NURSING MOTHER

REDUCED EXPRESSION OF ANTIMICROBIAL PEPTIDES AND MUCINS IN THE NOD MOUSE PRECEDES THE COLONIZATION OF A DIABETES PROMOTING MICROBIOTA

LIST OF FIGURES (Continued)

Figure *Page*

UNPUBLISHED DATA

LIST OF TABLES

Table *Page*

INTRODUCTION

REDUCED EXPRESSION OF ANTIMICROBIAL PEPTIDES AND MUCINS IN THE NOD MOUSE PRECEDES THE COLONIZATION OF A DIABETES PROMOTING MICROBIOTA

UNPUBLISHED DATA

INTRODUCTION

Type 1 Diabetes

The importance of the gastrointestinal (GI) system in shaping systemic immunity and in influencing autoimmune disease has become a major area of research [1-7]. One such disease that may have a GI link is type 1 diabetes (T1D). T1D is classically defined as immune mediated destruction of insulin-producing pancreatic β-cells [8]. Since its discovery in 1921, insulin remains the most effective treatment for T1D patients [8]. An estimated $15 billion is spent yearly in the United States on medical treatment for patients, with the disease presenting a heavy burden on the health care system [8]. Many avenues are being pursued in treating T1D, one of which is manipulation of the gut microbiota. The observation that initiated the investigation of a link between T1D and alterations in the GI ecosystem was the presence of serum antibodies to cow's milk protein, a luminal antigen, in patients with the disease [9]. A second observation that linked the GI ecosystem and T1D is the apparent protection from disease in infants that have been breastfed [10]. However, the strongest link may be the correlation between Celiac disease (CD; a autoimmune disorder due to immune reactivity to foods containing gluten) and T1D, as 3-6 % of all patients with T1D also have CD [11]. The common link between these three findings is the presence of systemic immune responses to GI luminal antigens/food proteins, which indicates a potential alteration in GI barrier function and a subsequent impact on the mucosal and systemic immune response. Furthermore, research has shown that the pancreatic lymph node (PLN) serves as a draining site for

luminal antigens of the GI system, strengthening the link between the GI ecosystem and T1D [12, 13].

Two common animal models used to study T1D pathogenesis are the non-obese diabetic (NOD) mouse and the BioBreeding diabetes prone (BBdp) rat. As future research explores the role of the GI system in T1D, the strengths of these models must be determined by understanding their similarities and differences in intestinal permeability, intestinal barrier function, commensal microbiota, and mucosal immune function when compared to human subjects [14]. The NOD mouse model has a disease progression similar to humans, as it develops peri-insulitis, followed by insulitis, and subsequently diabetes. The NOD mouse also contains the most common polymorphisms in major histocompatibility complex antigens that are associated with T1D in humans [15, 16]. Like the NOD mouse, the BBdp rat progressively develops more severe insulitis throughout its life and then develops diabetes[17]. BBdp rats also have the same MHCII polymorphism that is associated with T1D in the NOD mouse and humans [18]. In humans the MHCII haplotypes DRB1, DQA1, and DQB1are associated with an increased risk to develop T1D, specifically with the replacement of the amino acid Asp at codon 57 with either Ala, Ser, or Val [19]. Both the NOD mouse and the BBdp rat have been reported to have a leaky gut, as is further described below [20-22]. Understanding these similarities and differences will also be important in determining what is contributing to disease pathogenesis and what controls should be used to properly study the disease.

Intestinal Barrier

The intestinal barrier is composed of multiple components, which together form a selective blockade. Small molecules and antigens can cross this barrier in one of two ways, the paracellular route or the transcellular route. The paracellular pathway allows the passage of small molecules that are <600 Da. Alterations in paracellular permeability have been studied extensively in intestinal disorders including CD, in which patients have been shown to have lowered epithelial electrical resistance compared to healthy patients, indicating increases in intestinal permeability [23]. In patients with active CD, elevated expression of the inflammatory cytokine IFN-γ is associated with decreased expression of tight junction proteins (TJPs), which leads to increased paracellular permeability. Paracellular permeability is also increased in T1D and pre-diabetic patients, as measured by the lactulose/mannitol assay (LA/MA ratio) [24, 25]. The transcellular pathway allows for the passage of larger molecules, even whole bacteria, through epithelial cells via a process that is also associated with an increase in IFN-γ [23]. Normally only 0.1% of the original antigen transcytosed reaches the serosal compartment, highlighting the effectiveness of an intact intestinal barrier [26]. Antigens that manage to avoid degradation in epithelial cell lysosomal compartments have the potential to be immunogenic [23, 26]. This can be modeled in CD patients, as patients with active CD given a gliadin monomer will have detectable levels of the antigen in their serum, while patients with inactive disease do not [23, 27]. These observations point to an alteration of the transcellular pathway leading to increased intestinal permeability in CD, while the majority of T1D data indicates an increase in paracellular permeability, with less known about transcellular permeability.

The gut epithelium, along with the paracellular and transcellular pathways, are not the only components of the intestinal barrier that prevent small molecules and antigens from crossing the barrier. Other factors that contribute to the concept of a selective barrier include the production of mucins and antimicrobial peptides (AMPs), innate and adaptive immunity, and the presence of luminal commensal microbiota.

Mucins are complex glycoproteins secreted by epithelial cells that function to prevent antigens from reaching the epithelium by forming a physiochemical barrier [14, 28]. Colon mucus is composed of two layers: i) a thin sterile layer that covers the epithelial surface and ii) a thick outer layer that contains commensal bacteria, AMPs, and IgA [29]. Damage to or alterations in the outer layer allows bacteria (commensal and pathogenic) to come into contact with epithelial cells, leading to inflammation [14, 29]. Probiotic strains of bacteria, such as *Lactobacillus plantarum*, have been shown to be capable of inducing mucin production and therefore preventing pathogenic *Escherichia coli* from breaching the intestinal barrier [28]. Along with mucin production, commensal microbiota are also essential for maintaining intestinal homeostasis through colonization resistance, preventing immunogenic antigens from reaching the epithelial barrier [30, 31]. The importance of a healthy microbiota can be seen in human subjects who have recently undergone a fecal transplant after suffering from *Clostridium difficile* infection. After fecal transplant the *C. difficile* bacteria is replaced with a normal microbiota and patient health is restored [32]. It has been speculated that one way commensal populations are maintained and regulated is through the production of AMPs [33]. These AMPs are the

primary defense against pathogenic bacteria and allow commensal populations to remain in their niche.

The final component of the intestinal barrier is the mucosal immune system. With its high continuous exposure to commensal microbiota and food antigens, the ability of the mucosal immune system to respond in a dampened or regulatory manner to luminal antigens is essential. To achieve this homeostasis, dendritic cells (DCs), innate lymphoid cells (ILCs), regulatory T cells (Tregs), and cytokines are all responsible for controlling the response of effector T-cells to commensal and food antigens that reach the intestinal barrier [34-38].

Intestinal Permeability

Intestinal permeability has been shown to play a role in multiple autoimmune diseases including CD and inflammatory bowel disease [23, 39]. The concept of a leaky gut has now been associated with T1D, as patients with T1D and their relatives have a more permeable intestine than controls [7, 20, 24, 40-42]. As early as 1986, data was gathered showing that patients with T1D excreted higher levels of urinary lactulose compared to healthy controls, indicating an increase in their intestinal permeability [40]. In the BBdp rat, although their permeability decreases with age, the diabetes prone BBdp rats have a higher level of intestinal permeability compared to the Wistar rat, that does not develop diabetes [21]. BBdp rats also have an increase in intestinal permeability compared to BioBreeding diabetic resistant rats (BBdr) rats, but there is no difference in colonic permeability between the two strains [43].

Permeability is measured in multiple ways in animal models and human patients. The levels of TJPs, which control paracellular permeability, can be assessed by western blot and PCR [21, 26, 44]. Paracellular permeability is determined by the pore size of tight junction proteins (TJPs), with different combinations of TJPs determining a large versus small pore size [26]. Two groups of TJPs, the Claudins (Cldn) and Occludin (Ocln) are considered to be master regulators of permeability due to their ability to open and close paracellular junctions [20]. High performance liquid chromatography can also be used to determine the lactulose/mannitol (LA/MA) ratio, which measures sugar levels in urine and serves as another indicator of paracellular and transcellular permeability [43, 45]. Mannitol is a small sugar that permeates the barrier through a transcellular pathway, while lactulose is a larger sugar that permeates the barrier through the paracellular pathway and the LA/MA ratio allows you to determine which permeability pathway is being affected [45]. The LA/MA ratio has been used in human subjects, where research has shown that diabetic patients have a higher LA/MA ratio compared to healthy controls [24]. Like lactulose and mannitol, FITC-dextran (MW ~4000) is another sugar that can be used to measure paracellular permeability in the small intestine of animal models. FITC-dextran is a fluorescent sugar that is given by oral gavage or enema and will leak across the gut if the intestinal barrier is permeable. Serum levels of FITC-dextran can then be measured by fluorescent intensity to determine the level of permeability [22, 46]. A natural indicator of intestinal permeability is zonulin levels in serum [47, 48]. Zonulin was first identified when Vibrio Cholera was found to produce zonula occludens toxin (Zot). Zot was shown to interact with TJPs and increase intestinal permeability [47]. A human analogue of Zot was identified named zonulin (also known as haptoglobin) and its

effects are localized to the small intestine [47, 49]. Another final method to measure barrier function is by giving mice an oral gavage of bacteria and then removing lymph nodes and culturing homogenate on agar plates and counting colonies to determine bacterial translocation [22]. All of these assays have been used in T1D to determine the role of permeability in disease pathogenesis.

At 12 weeks of age NOD mice have increased intestinal permeability compared to the non-obese diabetic-resistant (NOR) and C57BL/6 strains of mice when using FITC-dextran as a measure of permeability [22]. In the NOD mouse, microbes can also lead to alterations in intestinal permeability, as indicated by increased serum FITC-dextran levels after *Citrobacter rodentium* infection [22]. The administration of *C. rodentium* alters the transcellular pathway and leads to a higher bacterial translocation of *C. rodentium* to the mesenteric lymph node (MLN) and the PLN of infected NOD mice when compared to C57BL/6 mice [22]. Both BBdp and BBdr rats have lower levels of the TJP Cldn1 compared to non-diabetic Wistar rats [50]. In rats that have been inoculated with *Lactobacillus johnsonii* there is a higher expression (mRNA) of the TJP Cldn1 in BBdp rats that do not have diabetes compared to BBdp rats that do have diabetes. Interestingly the expression (mRNA) of the TJP occluding (Ocln) decreases in diabetes free BBdp rats compared to healthy controls and diabetic BBdp rats [44], perhaps indicating that claudin-1 (Cldn1) is the master regulator of intestinal permeability in diabetic strains of rats. In contrast to the BB rat, limited data on human subjects shows an increase in mRNA expression of Cldn1 from intestinal biopsies of diabetic patients compared to health controls [24]. The discrepancies in Cldn1 expression between BB rats and humans

indicates that the role of TJPs during the pathogenesis of T1D may differ between species and requires further investigation.

Past research has shown the short chain fatty acids (SCFAs) produced by bacterial fermentation of carbohydrates in the colon can affect permeability [51]. Permeability in the BBdp rat can be altered by direct administration of the SCFA, sodium butyrate, to rats between 10-23 days of age, leading to a decrease in distal colon permeability compared to control rats [52]. Butyrate is most likely altering TJPs as it has been shown to play a role in proliferation, differentiation, apoptosis, and maintenance of tight junctions in epithelial cells [52]. In C57BL/6 mice, butyrate has also been shown to increase insulin sensitivity and may be working in conjunction with the beneficial effects on TJPs seen in the BBdp rat to prevent T1D [52, 53]. Current data shows the importance of intestinal permeability in T1D disease models and implies that exploration of potential therapeutics to decrease intestinal permeability in the pre-diabetic state may be worthy of future exploration.

In inflammatory diseases of the gut, such as CD, patients have higher zonulin levels in their intestinal submucosa compared to control patients [54]. Increases in zonulin have also been shown to precede the development of T1D in the BBdp rat [48]. In BBdp rats luminal levels of zonulin increase between 20-70 days when compared to BBdr control rats or BBdp rats that do not go on to develop T1D [48]. The effects of zonulin can be prevented with a zonulin inhibitor (FZI/0), which lowers the incidence of T1D by 50% in the BBdp rat [48]. In humans, not only have zonulin levels been shown to be increased in TID patients, but levels are also elevated in relatives of T1D patients compared to healthy controls [24].

Diet has been shown to reduce T1D incidence in animal models of the disease [43, 55-58] The effects of diet on permeability are still under investigation with some research pointing to a decrease in permeability, while other data points to no change in permeability [43, 45]. A hydrolysed casein (HC) diet is able to restore intestinal integrity, as measured by the LA/MA ratio, while also lowering zonulin levels in the BBdp rat [45]. HC diets lead to a 60% decrease in the diabetes incidence in BBdp rats, when compared to BBdp rats on a control diet [43]. BBdp rats on a HC diet have elevated levels of Cldn1 expression in the ileum leading to a reduction in permeability, this reduction in permeability may be increasing overall intestinal health, which leads a reduction in T1D incidence [45]. Unfortunately, the effect of diet on epithelial permeability in the NOD mouse or in human patients has not been thoroughly investigated. Importantly, alterations in the intestinal microbiota induced by dietary alterations need to be further explored in both animal models and humans, as it is clear that microbial components can have direct effects on the intestinal barrier.

In many mouse models, physiological β-cell death occurs at 2wks of age, at which time lymphocytes dectect antigens that will lead to their activation and eventual disease pathogenesis [59]. Luminal antigens may trigger these diabetogenic lymphocytes to subsequently recognize and/or actively respond to pancreatic antigens at this 2 week time point. It is likely the permeable intestinal barrier would allow for the exposure to these luminal antigens. The pathway that these luminal antigens take to activate lymphocytes may be even more direct than just simple leaking. Luminal antigens are also able to drain into the PLN, where they could potentially activate lymphocytes that have access to the

pancreas [60, 61]. These activated lymphocytes could then cause β-cell damage leading to T1D. Barrier permeability will continue to be an important area of research in T1D because of the potential role of antigens leaking across and activating the immune system, which could lead to pancreatic β-cell death.

Antimicrobial Peptides and Mucins

Additional factors that contribute to barrier function are mucins produced by goblet cells and enterocytes, and AMPs produced by enterocytes and Paneth cells within the intestinal tract. Human and rat neutrophils are also capable of producing defensins, a class of AMPs, while murine neutrophils cannot [62, 63]. Mucins are complex glycoproteins that protect mucosal epithelial cells either through steric hindrance, forming a physiochemical barrier, or through specific mucin-bacteria interactions [28]. In the human, mouse, and rat intestine the majority of the secreted mucin layer consists of MUC2 or its equivalent, while MUC1, MUC3A/B, MUC12, MUC13 and MUC17, or their equivalents, are expressed on the epithelial cell surface [14, 64, 65]. Goblet cells also produce a small peptide known as trefoil factor 3 or intestinal trefoil factor (TFF3/ITF) [65]. TFF3 is thought to have the ability to increase the viscosity of MUC2 by stabilizing the mucin and thus helping to protect the epithelial cell surface [65]. Interestingly TFF3 has also been shown to induce β-cell proliferation in rat and human islets [66]. TFF3 is not the only trefoil factor that could potentially affect T1D. Trefoil Factor 2 (TFF2, also known as spasmolytic polypeptide) is secreted by gastric mucous neck cells and has been shown to induce β-cell proliferation in mouse islets [67]. The

interaction of trefoil factors with mucins in diabetes is still an unexplored area, but could represent a therapeutic pathway to alter disease incidence.

One main type of AMPs is the defensins, which are cationic peptides that kill bacteria by disrupting the bacterial cell membrane. There are two main classes of defensins, α and β [68]. α-defensins are primarily expressed in the small intestine by Paneth cells located in the crypts, but can also be produced by neutrophils and macrophages [69, 70]. β-defensins are expressed by almost every epithelial cell in the small intestine and colon, but there is little expression in the crypts of the small intestine [71]. In mice and rats epithelial defensins are referred to as cryptdins [72, 73]. Both α and β-defensin expression have been shown to be attenuated in the ileum and colon of patients with inflammatory bowel disease, however the role of defensins in T1D is still widely unknown [74]. One recent report has indicated that a specific genotype of defensin β-1 in patients with T1D may result in decreased defensin protein production [75]. Another AMP that is primarily synthesized in the colon is cathelicidin (CRAMP) [76]. Studies in the BBdp rat have shown that a HC diet (which lowers the incidence of T1D) upregulates the gene expression of CRAMP in the ileum and jejunum [56]. In mice, CRAMP has been shown to regulate pathogenic *C. rodentium* colonization and in humans it regulates pathogenic *E. coli*, indicating that some of its effects may be via its ability to shape the luminal microbiota [76]. Like the defensins, there is still much that is unknown about the role of CRAMP in the progression and pathogenesis of T1D. The importance of AMPs and mucins in shaping the microbiota is widely accepted, but their role in disease pathogenesis is still not understood. Interestingly it is unknown whether bacterial colonization is dictating expression of AMPs and mucins or whether AMP and

mucin expression dictates what bacteria are able to colonize the GI system. As research continues to uncover the role of the microbiome in T1D, AMPs and mucins will emerge as an important experimental focus.

When BBdp rats develop diabetes they have a lower number of goblet cells when compared to BBdr rats and BBdp rats that have been treated with *L. johnsonii* (which delays T1D development) [44]. This indicates that mucins play a protective role in diabetes and may be lowering the incidence of disease and that specific strains of bacteria can alter mucin expression. When the Lactobacillus strains Lp299v and LrGG are co-incubated with the colonic cell line (HT29), an increased expression of MUC3 mRNA can be detected in the HT29 cells [28]. This increased MUC3 production then inhibits the adherence of pathogenic E. coli to HT29 cells [28]. This inhibition of bacterial adherence can prevent bacterial antigens from crossing the intestinal barrier and activating the immune system, which could lead to an altered incidence of diabetes. Interestingly, a type 2 diabetes study revealed decreased disease incidence after daily consumption of yogurt with active cultures over a 10-30 year period [77]. However, as the study did not investigate if there was an alteration to the commensal microbiota of the participants, the mechanisms of the effect is unknown. An interesting observation pointing to a link between diabetes and mucins has been seen when using the anti-inflammatory drug dexamethasone. Dexamethasone has been shown to inhibit mucin production in mice, while a separate study has shown that dexamethasone also leads to an increase in the incidence of diabetes in rats [78, 79]. One possible mechanism may be the reduction of mucin, which leads to increased antigen exposure, and alters the immune response leading to diabetes. However, as dexamethasone also directly suppresses the

immune response, the critical effect of this drug in T1D is unclear. The importance of mucins has also been linked to the development of T1D in humans, as patients with T1D are reported to have a lower level of lactate and butyrate producing bacteria, which lead to a decrease in mucin synthesis and allow the intestinal epithelium and the mucosal immune system to be exposed to an increased number of luminal antigens [80].

Diet can also regulate mucin and AMP production. BBdp and BBdr rats on a plant based diabetes promoting diet (NTP diet) have reduced levels of mucins, with a more dramatic affect seen in the diabetic BBdp rat [55, 81]. In addition, BBdp rats that are fed the T1D-protective HC diet, have an increase in the AMP CRAMP compared to cereal fed rats [56]. Not only is diet affecting mucin production but it also affecting AMP production and together these two changes in GI barrier function are associated with an alteration T1D incidence.

Surprisingly little research has focused on manipulating AMPs and mucins to control the microbiota and alter disease incidence. Two potential agents that could be used are bethanechol and vanadate. Vanadate has been used to treat T1D in NOD mice, diabetic rats and humans, where it has been shown to lower blood glucose and reduce insulin dependence [82-85]. Importantly vanadate has also been shown to increase MUC1 shedding in human uterine epithelial (HES) cells and increase MUC5AC expression in human airway epithelial cell lines and the airways of mice [86, 87]. The role of vanadate in the intestine is not known, but may serve as an agent to increase mucin expression in the gut. Bethanechol, a cholinergic agonist, is closely related to carbamylcholine (Cch), which itself has been shown to have ex-vivo antimicrobial activity through the stimulation of Paneth cells [72, 88]. Like Cch, bethanechol has been

shown to induce Paneth cell secretions, and it also causes an increase in expression (mRNA) of metalloproteinases, including matrix metalloproteinase enzyme matrilysin (MMP7). MMP7 is needed to cleave defensins produced by Paneth cells from their pre to their active pro configuration [71]. However, the antimicrobial activity of these secretions after stimulation with bethanechol has not been investigated [89, 90]. Bethanechol, like vanadate, when given subcutaneously (1-2 ug/g body weight) has been shown to lower the blood glucose levels of NOD mice [91]. Future investigation is required to determine if these drugs are improving the outcome of T1D due to an alteration in the epithelial barrier and/or commensal microbiota.

Mucosal Immunity

Over 15 years ago, the observation was made that the jejuna of patients with T1D showed increased α4β7+ cells in their intestinal lamina propria, a sign of immune activation[92]. Cells expressing these same mucosal addressins and integrins are components of islet-infiltrating T-cells, implying a link between the mucosal immune system and the pancreas [93, 94]. Intriguingly, it has now been shown that the pancreatic lymph nodes (PLNs) can sample not only self-antigens from the pancreas, but are also exposed to microbial products from the GI tract [61]. In addition, in young NOD-mice, the cells with the most diabetogenic potential were primarily found in gut-associated lymph nodes [95]. In a normal mouse (or human), there appears to be tight mucosal compartmentalization of microbial antigens by the mucosal immune system, which limits this systemic exposure [96]. However, in animals/humans who have altered intestinal barrier permeability (T1D), the microbial products can freely cross the

intestinal barrier and reach draining lymph nodes, including PLNs [97]. This has clearly been demonstrated in patients with T1D, where systemic immune responses to cow's milk proteins have been observed [98].

In addition to these draining lymph nodes, there are two other lymphoid components in the gastrointestinal immune system, the lamina propria and intraepithelial lymphocytes (IELs) [99]. It has been reported that a deficiency in IELs occurs prior to the onset of diabetes in BBdp rats and that neonatal thymectomy (which depletes both natural Tregs and IELs) in NOD mice accelerates diabetes [100, 101]. Transfer of IELs into thymectomized NOD mice can prevent diabetes, indicating that these mucosal immune cells play a key role in maintaining tolerance to pancreatic antigens [100]. Decreased numbers of Tregs in the lamina propria is a consistent finding in the small intestine of both patients with T1D and in NOD mice [102, 103]. However, it is unclear if this decrease is primarily in natural Tregs (derived from the thymus) or in induced Tregs. Data from NOD mice fed a diabetes-inducing gluten-containing diet shows a reduced number of Tregs and lamina propria dendritic cells, and T1D patients appear to lack the ability to induce Tregs, implying that there may be defects in both types of Tregs [102, 104]. However, alterations in Treg populations are not the only mucosal immune abnormalities noted in T1D. Besides Tregs, $CD4^+$ cells can also differentiate into Th1, Th2, and Th17 cells. Th1 cells contribute to the pathogenesis of T1D by producing IFNγ, which can activate and recruit cytotoxic $CD8^+$ T-cells and macrophages to the pancreas where they can destroy β-cells. Th2 cells produce cytokines, IL-4 and IL-5, which can stimulate B cells to produce IgM and IgG1, which may be involved in T1D pathogenesis [105]. The mechanism by which Th17 cells contribute to T1D pathogenesis is less clear.

Neutralization of IL-17 in NOD mice has been shown to reduce disease incidence and it has also been shown that Th17 cells can acquire a Th1 like phenotype and produce IFNγ [106]. Colonic T-cells in NOD mice express higher levels of both IL-17 and IFNγ and MLNs have increased Th17 and Th1 effector T cells [58, 102, 107]. In addition, children with T1D have been shown to have upregulaton of Th17 cells in peripheral blood [106]. However, the role of IL17 in T1D is controversial, as it has also been shown that BBdp rats that are rendered diabetes-resistant by treatment with *L. johnsonni* strain N6.2 have a Th17 bias [108]. One effect of this low-level intestinal inflammation is the increased crypt depth (reflecting increased proliferation) that is seen in NOD mice and BBdp rats [21, 58]. This crypt dept can be increased further in BBdp rats fed a diabetogenic diet [109].

In addition to the adaptive immune response in the GI tract, the innate immune system is also regulating the GI ecosystem through the secretion of IgA. Secretion of IgA protects the epithelium from insult by neutralizing bacteria in the GI lumen [110]. Due to its role in protecting the GI epithelium, IgA may have an important role in T1D pathogenesis. A study by Sayarifard et al. found that a small portion of T1D patients had significantly lower leves of serum IgA [111]. Another study by Satorres et al. found that patients with T1D have higher levels of *Staphylococcus aureus* IgA in their serum compared to healthy controls [112]. However, the GI ecosystem is a stable alliance between resident microbiota, immune mediators, and the epithelial barrier [99] and it is clear that diet and the environment can directly alter the microbiota. Therefore, it is difficult to ascertain what effects are due to mucosal inflammation and what are secondary to altered composition of the resident microbiota.

Microbiota

Recent reviews have emphasized the importance of microbiota in affecting the disease incidence of T1D across species [44, 113-122]. It was originally thought that germ free NOD mice developed diabetes at a higher incidence rate than mice with intestinal flora [123]. However, recent reports have found that germ free animals develop T1D at the same rate as specific pathogen free (SPF) mice and that it is the presence of certain bacteria that is needed to prevent diabetes [102, 124]. Human data has indicated that healthy subjects have a higher percentage of mucin-degrading bacteria from the generas Prevotella and Akkermansia, compared to T1D subjects [80]. This is in contrast to data in the BBdr rat that shows a reduction in mucin increases T1D susceptibility [55, 81]. These different results regarding mucin levels may be explained by variations in commensal populations and differences in immune responses to antigens between diabetic and non-diabetic subjects. Researchers have also followed children that are at a high genetic risk for developing T1D and shown that they have a decrease in intestinal microbiota diversity compared to controls [125]. These children at risk for T1D also have an increase in Bacteroidetes and a decrease in Firmicutes compared to age matched healthy controls [125].

In NOD mice, segmented filamentous bacteria (SFB), have been shown to cause an expansion of Th17, shifting away from a Th1 response, which is associated with disease [126]. Interestingly if anti-IL-17 is given to NOD mice starting at 10 weeks of age there is a decrease in T1D incidence; however, if the same treatment is given starting at 5 weeks of age there is no effect on disease incidence [127]. This suggest that perhaps SFB expression is transient at different stages of life, which could explain why anti-IL-17

treatment is only protective at 10 weeks of age in the NOD mouse. The potential role of SFB in altering T1D incidence BBdp rats is still unknown, but data has shown that as these rats progress towards diabetes their percentages of circulating IL-17 producing T-cells increases compared to BBdr rats [128]. Human patients with T1D have a higher number circulating IL-17 cells compared to recent onset and healthy control patients [129]. Diabetic subjects also have a higher percentage of Th17 along with defective Tregs in their pancreatic lymph node compared to healthy subjects [130]. Much like the BB rat, the SFB colonization status of patients with and without T1D has yet to be investigated. Although the role of Th17 cells in diabetes is not yet clear, varying levels of IL-17 amongst patients and control subjects indicates that there is most likely a difference in SFB colonization (or in colonization of a similar microbiota that can induce IL-17 production) between healthy and diseased groups.

The impact of microbiota on diabetes incidence is evident in experimental models that have been treated with antibiotics. In NOD mice the incidence of T1D can be significantly lowered when mice are treated with antibiotics [121, 131]. One of these antibiotics is vancomycin, which targets gram-positive bacteria. When administered to NOD pups at birth until weaning, vancomycin not only led to a depletion of gram-positive bacteria, but also surprisingly led to a depletion of gram-negative bacteria in the gut of the NOD mouse [121]. The reduction of both gram-positive and gram-negative bacteria leads to the emergence of *Akkermansia muciniphila*, which correlates with a lowering in the incidence of T1D [121]. Similar effects of antibiotics on the incidence of T1D in the BBdp rat have also been reported [120, 132]. BBdp rats given a combination antibiotic treatment of sulphamethoxazole, trimethoprim, and colistine sulphate to reduce

gram-positive and gram-negative bacteria have a lowered incidence of T1D [120]. The antibiotic-treated BBdp rats also have a reduced level of Bacteroides compared to rats that did develop diabetes [120]. Although the microbiota was not examined, treatment of BBdp rats with the antistaphylococcal drug fusidin also reduced the incidence of T1D in treated groups [132]. Interestingly in humans treated with a variety of antibiotics it has been shown that their mucin profile resembled that of a germfree rat, which differed from the mucin profile associated with the presence of gut microbiota as shown by analyzing mucins isolated from human subjects and germfree rats on electrophoresis gels and comparing banding patterns [133]. It was speculated that antibiotics were altering mucin profiles by removing mucin degraders Bacteroides, Ruminococcus, and Bifidobacterium from the GI system and we propose that this could be another contributing factor to antibiotics altering the incidence of diabetes [133].

The importance of specific commensal microbiota influencing T1D incidence has been most widely studied in the NOD mouse. The administration of the probiotic mixture VSL#3 (which contains multiple species of bifidobacteria, multiple species of lactobacillus, and *Streptococcus salivarius*) to NOD mice lowers the incidence of T1D [134]. NOD mice that are given VSL#3 have increased levels of IL-10 in Peyer's patches, the spleen, and the pancreas, likely leading to suppression of effector T-cells in these areas [134]. When administered to Wistar rats, VSL#3 also induced an increase in colon MUC2 expression, which could further explain the protective nature of VSL#3 in diabetes [135]. VSL#3 also has been shown to increase human β-defensin 2 in Caco-2 cells and this increase in AMPs represents another possible mechanism by which VSL#3 helps lower the incidence of diabetes [136]. In a recent study, NOD male commensal

microbiota were generally shown to have higher levels of Firmicutes and lower levels of Bacteroidetes compared to NOD female mice [119]. This difference in microbiota between male and female NOD mice resulted from a higher level of circulating testosterone in male mice, which correlates with a decrease in insulitis resulting in a decreased incidence of T1D in the NOD male mouse compared to the females [119]. Based on this observation, the incidence of T1D in female mice was lowered by giving them male cecal microbial content by oral gavage, which led to the colonization of the female intestine with male microbiota and a rise in testosterone levels [119]. In the BBdp rat the incidence of diabetes can be lowered when *L. johnsonii N6.2* is given twice (1d and 21d) to pups by oral gavage [44]. The administration of *L. johnsonii N6.2* results in an increase in mRNA levels of the TJP Cldn1 and decrease in Ocln. Along with alterations in TJPs, BBdp rats that are given *L. johnsonii N6.2* have decreased levels of the inflammatory cytokines TNF-α and IFN-γ, which indicates a shift in the mucosal and systemic immune response [44]. It has also been shown that in BBdp rats that do not develop T1D there is an increase in Lactobacillus populations in the ileum [44].

The use of antibiotics and direct administration of bacteria are not the only ways to potentially affect the microbiota and alter the incidence of T1D. Wolf et al. has shown that female NOD mice on acidified H_2O (AW) have an increase in Firmicutes and a decrease in Bacteroidetes compared to NOD mice in the same colony on neutral H2O (NW). This shift in microbiota correlates with a decrease in the incidence of T1D in female NOD mice on AW compared to those on NW [137]. However, a second group has reported that in their animal facilities, the NOD mice on AW have a ileal commensal population that is 98% Lactobacilli; and when the NOD mice are on NW this percentage

drops to 78% [122]. This microbial composition led to an onset of T1D that was much more rapid in NOD mice on AW compared to NW. This is contrast to what was reported by Wolf et al. There are several potential explanations for these differing results. First, in the second study (Sofe, et.al.) the mice on NW had a higher percentage of *Eubacterium bioforme*, which may be contributing to a delayed onset of T1D [122]. However, more likely is the concept that the observed variance in T1D incidence (when comparing mice on AW or NW between Wolf, *et. al.* and Sofi, *et. al.*) is a function of the differences in baseline microbiota between the two institutional animal facilities, which appear to have different ratios of Firmicutes and Bacteroidetes between the water groups. Therefore the real conclusions that can be made from these observations are that dietary alterations in microbiota clearly have an impact on the incidence of T1D and imply that these types of manipulations deserve further study.

Manipulation of the microbiota has proven to be an effective way to treat T1D in animal models as mentioned above. Normalization of the microbiota is very difficult, as seen by a 30% variance in the microbiota in the same strain of mice housed in the same facility in different cages [138]. Research has also shown the importance of the mother's microbiota regardless of genetic background as seen in homozygous and heterozygous pups born in the same litter [139]. With observations such as these, the question remains of how can we effectively and efficiently change the microbiota from disease promoting to disease preventing. To date current protocols that allow for the manipulation of microbiota (including, antibiotics, gavaging of fecal content, co-housing, and cross-fostering) all require daily treatments and large number of animals and are therefore

inefficient [121, 131, 140-142]. Methods are needed that will effectively shift the microbiota early that will last for the lifetime of the mouse, protecting them from disease.

Aim of Dissertation

This dissertation will examine intestinal permeability, barrier function, commensal microbiota, and mucosal immunity and the role they play in T1D. Animal models are currently the best mode of studying the mechanism of T1D pathogenesis. With an increased understanding of the role of the gut in T1D it is important to have a clear understanding of the similarities and difference between animal models and human subjects. Several mouse strains commonly used do not have abnormalities within their GI ecosystem and fail to address this important area of T1D pathogenesis. This dissertation will examine the difference between the widely used NOD mouse and the NOR mouse. Specifically we will examine the role of AMPs and mucins and their ability to influence disease promoting and disease preventing commensal microbiota. We will also examine the ability of commensal microbiota from NOR mice to lower the incidence of T1D after transfer into NOD mice through cross-fostering. We hypothesize that both NOD and NOR mice have alterations to their GI ecosystems and it is these alterations that determine disease incidence in NOD mice and protection from disease in NOR mice.

Table 1: *Alterations in Gastrointestinal Permeability in Patients and Models of Type 1 Diabetes*

Measure of Permeability	NOD	BBdp	Human
Tight Junction Proteins (TJPs)	Variations in TJPs compared to non-diabetic mice is not known	Lower protein expression of Cldn1 compared to Wistar rats (24) Inoculation with *L. johnsonii* increased mRNA expression of Cldn1, but decreases expression of Ocln (31)	Increased mRNA expression of Cldn1 from intestinal biopsies of diabetic patients (9) Decreased mRNA expression of Cldn2 from intestinal biopsies of diabetic patients (9)
Lactulose/mannitol (LA/MA)	Not commonly used to measure permeability in NOD mice	Lower LA/MA ration in BBdp rats that are on a diabetic resistant diet (32)	Higher LA/MA ratio in T1D patients compared to healthy controls (9,27)
FITC-dextran	Increased serum levels of FITC-dextran compared to NOR and C57BL/6 (25)	Not commonly used to measure permeability in BB rats	Not commonly used to measure permeability in human subjects
Zonulin	Zonulin levels in NOD mice have not been reported	High levels of circulating zonulin precede T1D (34) Zonulin inhibitor lowers the incidence of T1D (34)	Zonulin levels are elevated in T1D patients (9) Non-diabetic 1st degree relatives of patients with T1D have increased zonulin levels (9)
Bacterial Translocation	Bacterial translocation increased to MLN and pancreatic lymph node after *C. rodentium* infection in NOD mice compared to C57BL/6 mice (25)	Bacterial translocation in diabetic BBdp rats has not been investigated	Bacterial translocation in diabetic subjects has not been investigate

Table 2: *Diet Effects on the GI Ecosystem in Models of Type 1 Diabetes*

Intestinal alteration	Diet Induced
Permeability	Short chain fatty acids (SCFAs) from the bacterial fermentation of carbohydrates decrease colonic permeability in BBdp rats (37). Hydrolysed casein (HC) diet restores intestinal permeability, as measured by LA/MA, zonulin levels, and Claudin 1 levels in the BBdp rat. Diabetes incidence decreased by 60% in BBdp rats (30,32).
Antimicrobial Peptides and Mucins	HC diet in the BBdp rat up regulates the gene expression of the antimicrobial peptide (AMP) cathelicidin (CRAMP) (43). The plant based diabetes promoting (NTP diet) reduces mucins at a greater level in the BBdp rat compared to the BBdr rat (42). Probiotic VSL#3 increases mucin (MUC2) expression in the colon of Wistar rats and increases expression of the AMP, β-defensin 2 in Caco-2 cells (98,99).
Mucosal Immunity	Gluten based diabetes promoting diet, leads to a decrease in Tregs in the lamina propria of NOD mice (72).
Microbiota	The probiotic VSL#3 lowers the incidence of diabetes in NOD mice (97). Acidified and neutral H_2O alters the microbiota in NOD mice and alters diabetes incidence (86,100).

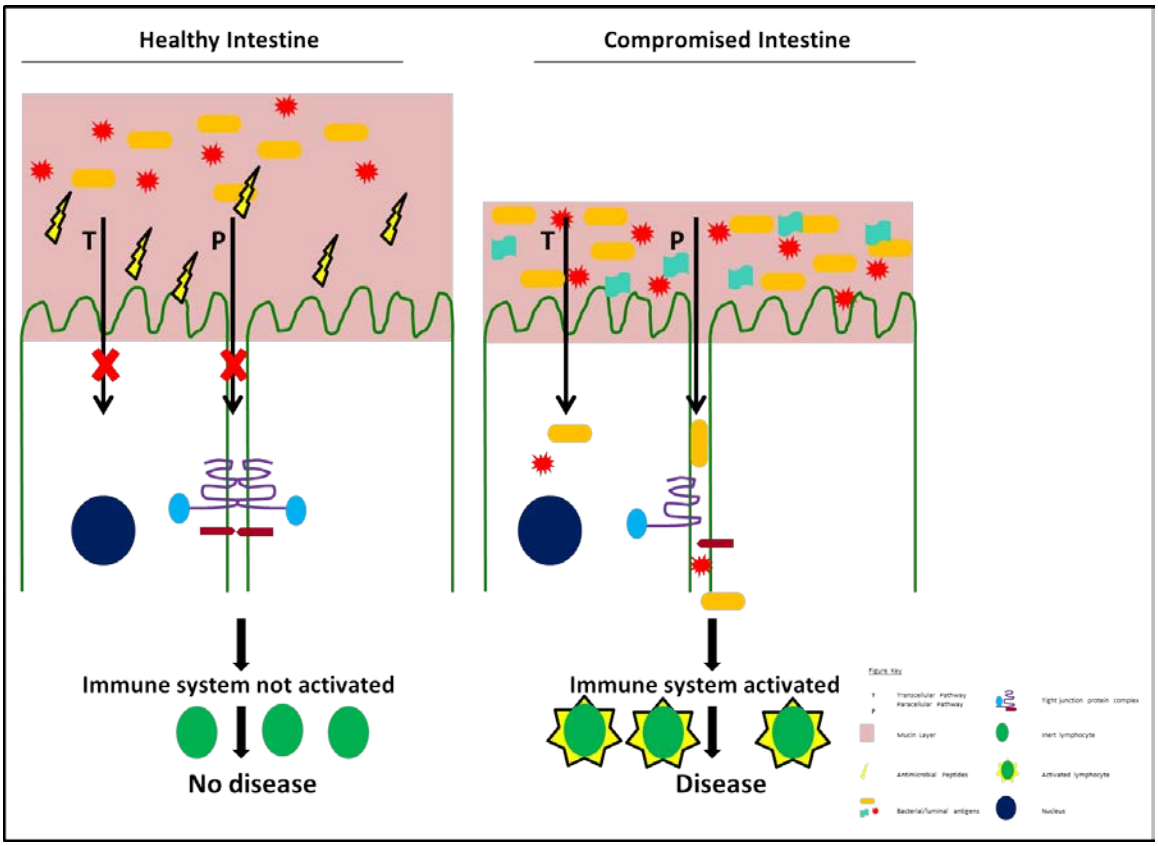


Figure 1. Intestinal homeostasis is maintained by a healthy microbial population, mucin layer, antimicrobial peptides, and tight junction proteins. Together these factors prevent or limit the exposure of bacteria and luminal antigens to the epithelium, which result in non-activated or tolerant immune system. In a diseased state when alterations are made to the microbial population, mucin layer, antimicrobial peptides, and tight junction proteins, bacterial and luminal antigens are able to cross the epithelial layer. Once bacteria and other luminal antigens have crossed the epithelial layer the immune system is activated leading to diseases such as type 1 diabetes.

CROSS-FOSTERING IMMEDIATELY AFTER BIRTH INDUCES A PERMANENT MICROBIOTA SHIFT THAT IS SHAPED BY THE NURSING MOTHER

JOSEPH G DAFT, TRAVIS PTACEK, RANJIT KUMAR, CASEY MORROW, ROBIN G LORENZ

Submitted to *Microbiome*

Format adapted for dissertation

Abstract:

Background

Current research has led to the appreciation that there are differences in the commensal microbiota between healthy individuals and individuals that are predisposed to disease. Treatments to reverse disease pathogenesis through the manipulation of the gastrointestinal (GI) microbiota are now being explored. Normalizing microbiota between different strains of mice in the same study is also needed to better understand disease pathogenesis. Current approaches require repeated delivery of bacteria, large numbers of animals, and vary in treatment start time. A method is needed that can shift the microbiota of predisposed individuals to a healthy microbiota at an early age and sustain this shift through the lifetime of the individual.

Results

We tested cross-fostering of pups within 48 hours of birth as a means to permanently shift the microbiota from birth. Taxonomical analysis revealed that the nursing mother was the critical factor in determining bacterial colonization, instead of the birth mother. Data was evaluated using bacterial 16S rDNA sequences from fecal pellets and sequencing was performed on an Illumina Miseq using a 251bp paired-end library.

Conclusions

The results show that cross-fostering is an effective means to induce an early and maintained shift in the commensal microbiota from weaning until 32 weeks. This will allow for the evaluation of a prolonged microbial shift and its effects on disease pathogenesis. Cross-fostering will also eliminate variation within control models by normalizing the commensal microbiota between different strains of mice.

Background

In recent years it has been appreciated in both animal models and human patients that there are healthy and disease promoting microbiota (1-3). This can be seen in diseases such as inflammatory bowel disease (IBD) and type 1 diabetes (T1D) (4-8). In order to study the effects of the microbiota in healthy and diseased subjects, research has focused on replacing or shifting diseased promoting microbiota to healthy microbiota, thus potentially reversing the diseased state. This has proven to be quite challenging because within healthy individuals there is considerable variation in the microbiota. For example, mice of the same strain, housed in different cages, have a diverse microbiota that can account for up to 30% of the variance seen in microbiome studies (9). These observations call into to question the results of studies that utilize animals that are caged separately. In order to properly control the microbiota in animal models, antibiotics, gavage of fecal content, co-housing, or cross-fostering have all been used (10-14). However, these experiments have multiple drawbacks, including the fact that they require repeated delivery of bacteria and a large number of animals because the microbiota already present is not easily displaced. Further confounding the field is the wide variance in microbiota between the same strains at different facilities. A well published example is the presence of segmented filamentous bacteria (SFB) in C57BL/6 mice ordered from Jackson Labs that is absent in C57BL/6 mice ordered from Taconic Labs (15). Differences can even be seen in colonies within the same facility (16, 17).

Recent data has revealed the importance of maternal transmission of microbiota in the colonization of their offspring. Experiments with toll-like receptor (TLR) knockouts and wild type mice born to TLR knockout mothers via a heterozygous breeding, reveal an identical microbiota between all pups regardless of TLR status (18). This finding

contradicts the previous conclusion that TLR signaling plays a role in dictating the microbiota as shown by Wen *et al.* who showed a difference in the microbiota between $MyD88^{KO/+}$ and $MyD88^{KO}$ mice (19). One potential reason for these differing results could be that these mice were derived from germ free mothers and bacteria was then introduced. Therefore mice were not initially colonized by the maternal microbiota because mothers were germfree. This emphasizes that variation in microbiota maybe due to differences in the microbiota of the nursing mothers, not due to the knockout state of the pups. Controlling for colonization of maternal microbiota by using the same mother for all pups allows for proper controls when comparing different genotypes on the same genetic background. However, it does not address the proper way to compare microbiota between completely different strains that by definition have to be born to strain specific mothers.

A second method to normalize bacterial colonization is co-housing. Co-housing can induce a change in the gut microbiota, but requires one recipient (receiving new microbiota) to be housed with 3 donors (giving microbiota), requiring a large number of animals and a high experimental cost (10). Another method that has been effectively used in mice to shift microbiota is the addition of fecal bacteria from diabetic resistant MyD88 deficient mice to drinking water (11). The administration of this fecal water for a period of 3 weeks to non-obese diabetic (NOD) mice causes an increase in *Lachnospiraceae* and *Clostridiaceae*, while leading to a decrease in *Lactobacillaceae*. This shift in microbiota correlates with a reduction in the incidence of diabetes, but this treatment cannot be started until mice are approximately 4 weeks of age (11). Therefore a better model is

needed that is cost effective and that can manipulate the colonization of microbiota at birth.

In humans, microbiota shifts are being induced by diet or in the case of patients with *C. difficile* patients, by fecal transplants. Dietary studies in humans have revealed that you can induce a shift in microbial diversity with a plant-based and animal-based diet. However, as soon as subjects are taken off of their respective diet, microbial diversity returns to pre-diet levels within days (12). It is hypothesized that fecal transplants lead to colonization by bacteria that occupy the niche of *C. difficile* preventing it from colonizing the gut; however, the exact mechanism and long-term effects are still unknown (20-22).

Two problems that exist with current protocols are that the microbial shifts are not permanent and that shifts are introduced too late prior to the development of the rest of the GI ecosystem. To properly study the effect of shifting the GI microbiota, a method must exist that induces a long term shift early in life. Currently it is hard to accurately determine the benefits of altering the composition of an individual's microbiota if these shifts are not stable or if they are not introduced until later in life.

To develop a method for inducing a sustained microbial shift, we propose cross-fostering as a means of efficiently and effectively inducing a sustained microbial shift. To test this hypothesis we designed an experiment that would induce a shift early and we postulated would remain for the entire lifespan of the test subjects. The NOD and non-obese diabetic resistant (NOR) strains of mice were used to explore whether it was possible to induce an early and permanent shift between different strains of mice. To induce a change in the microbiota as early as possible, newborn pups from NOD and

NOR mothers were cross-fostered unto the opposing strains. Cross-fostering is the switching of newly born pups to non-birth mothers who themselves have recently had pups or are ready to nurse. The pups were nursed by mothers of the opposite NOD and NOR strains until weaning. At weaning pups were separated based on sex, but not strain, and feces was collected from pups and mothers for microbiome analysis by sequencing of the 16S rDNA gene using next-generation sequencing (Illumina MiSeq). When the study ended at 32 weeks, feces were again collected from the previously cross-fostered mice for microbiome analysis. Comparison of bacterial phyla was then made between mice at weaning and the end of the study. This analysis of microbiota at 4 weeks and 32 weeks will determine if cross-fostering causes a microbial shift to resemble the nursing mother and it will also determine if this shift is temporary or permanent.

Methods

Animals

NOD/ShiLtJt mice and NOR/LtJ mice originally obtained from Jackson Laboratory (Bar Harbor, Maine) were bred and maintained under specific pathogen-free (SPF) conditions. NOD (n=8) and NOR (n=8) mice were used for this experiment and the experiment has been repeated with NOD (n=12) and NOR (n=16) mice (data not shown). All animals were housed in Thoren Isolator ventilated racks (Hazelton, PA). All caging, bedding, and food were sterilized prior to use. Both NOD and NOR mice were put on acidified water within a pH range of 3-3.5. Water was acidified using 1N HCl. Mice were fed autoclaved NIH-31 rodent diet (Harlan Teklan, Madison, WI) ad libitum. The Institutional Care and Use Committee of the University of Alabama at Birmingham approved all experiments. A detailed list of our facility's SPF conditions can be accessed at http://main.uab.eud/sites/ComparativePathology/surveillance/.

Cross-fostering

Breeding pairs of NOD and NOR mice were simultaneously set up when individual mice reached approximately 6 weeks of age. Only pups born to NOD and NOR breeding pairs within 48 hours of each other were used for cross-fostering. After both NOD and NOR litters had been born, half of each litter was removed and put with the mother of the opposite strain. Litters then contained pups born to that nursing mother and pups of the opposite NOD or NOR strain (Fig. 1). Fostered pups were marked daily with a sharpie on the back of the neck until their ears were able to be clipped (approximately 7 days) for identification purposes. The pups were nursed by their respective mothers until weaning. At weaning pups were separated based on sex, but not

strain, and feces was collected from pups and mothers and stored at -20^{0}C until analysis. When the study ended at 32 weeks feces was again collected from the previously cross-fostered and control mice and microbial DNA was isolated from mouse fecal material.

Sample prep,sequencing and analysis

Fecal DNA was isolated using a ZR Fecal DNA MiniPrep™ kit as previously described (23). The V4 region of the 16S rRNA gene was amplified, and the amplicon was sequenced using an Illumina Miseq. The amplicon was 255bp and the sequencing library consisted of 251bp paired end reads. Sequencing was performed at the UAB Heflin Center for Genomic Sciences, with an average of 83354 reads per sample.

Microbiome amplicon libraries were analyzed using the Quantitative Insight into Microbial Ecology (QIIME) suite version 1.7 (24, 25). For analysis we used a wrapper for QIIME called QWRAP. Analysis with QWRAP was performed as previously described (23). Prior to analysis with QWRAP, we merged the overlapping forward and reverse reads using the fastq_mergepairs tool from the USEARCH package (26). Read pairs with more than 5% mismatches were discarded. This QC method handled the issue of low-quality read tails and replaced the QC metrics found in (21). Version 13.8 of the Greengenes 16S rRNA database was used for taxanomical assignment of OTUs at an 80% confidence threshold. Output for QWRAP includes taxa summary tables (frequency of all given taxa by sample, by taxanomic level), alpha diversity measurements (chao1, PD whole tree, Shannon, Simpson), and distance matricies and principle coordinate analysis plots for beta diversity.

Statistical analysis

Statistical analyses of differences between groups were performed using QIIME's built in stats packages. To determine overall differences between the microbiomes of different groups, we tested for significant differences ($p<0.05$) in beta diversity using compare_categories.py using the PERMANOVA test. The distances matrices used for this test were the same used earlier in the pipeline for PCoA plots, generated by beta_diversity.py using both the weighted and unweighted unifrac metrics. To identify differences between groups at the taxanomical level, we tested for significant differences between groups in the average proportion for each taxa and in the presence/absence of each taxa using otu_category_significance.py with ANOVA and g_test, respectively. The paired-T test was used for comparisons of taxa proportions between two different timepoints. Due to the large number of tests (one per taxa), a p-value was considered significant if it was <0.05 after false discovery rate (FDR) correction. The taxa summary tables used for these analyses, generated by summarize_taxa.py, were filtered for OTUs with a frequency of less than 0.0005%.

Results

Nursing mother, not birth mother, determines fecal microbiota composition

The relationships between microbial communities in NOD and NOR mice that had been nursed by either NOD or NOR mothers were visualized by phylogenetic analysis using PCoA plots using both the unweighted unifrac distance matricies (Fig. 2). Four distinct groupings were seen based on nursing mother (not birth mother) and age. The grouping is also clear when visualizing the distance matrix of the samples (which was used to generate the PCoA plots) as a phylogenetic tree (Fig. 3). Both the PCoA plots and phylogenetic tree are visualizations of β-diversity, which was significantly different between all four clusters ($p<0.001$ for clustering by age and by nursing mother). Clustering by nursing mother and by age was also significant when using the weighted unifrac distance matrix ($p=0.002$ for both nursing mother and age, PCoA plot not shown). Significant statistical differences in α-diversity between mice nursed by NOR or NOD mothers was not seen ($p>0.05$,). Feces from 4-week old NOD and NOR pups nursed by a NOR mother have microbiota resembling that of NOR mice, while feces from 4-week old NOD and NOR mice nursed by a NOD mother have microbiota resembling NOD mice. This is seen at weaning and at 32 weeks when the study ended. It is important to note that the groupings shift between 4 and 32 weeks, but even at 32 weeks mice are still grouping based on nursing mother and not birth mother.

Mice nursed by NOD and NOR mothers have different intestinal microbiota

To compare the intestinal microbiota of NOD and NOR fostered mice, we first examined the relative proportions of bacterial phyla in these mice (Figure 4). Because the samples showed significant differences in clustering by age, analyses were stratified by

age. At 4 weeks, the average proportions of Bacteroidetes, Firmicutes, Tenericutes, Verrucomicrobia and candidate division TM7 were significantly different in NOD and NOR nursed mice and at 32 weeks, the average proportions of Tenericutes and TM7 were significantly different. At both time points, Tenericutes and TM7 was elevated in NOR nursed mice, while at 4 weeks, Firmicutes were elevated in NOR nursed mice and Bacteroidetes were elevated in NOD nursed mice.

The quality of our sequencing allowed us to further resolve the differences in composition of intestinal microbiota of NOD and NOR mice nursed by NOD mothers at the genus level. Genera with a statically significant difference by either ANOVA (difference in quantity) or G-test (difference in presence/absence) after FDR correction were selected. Like analyses at the phylum level, these analyses were stratified by age. At 4 weeks of age, NOR-nursed mice had higher proportions of *Prevotella, Parabacteroides, Sutterella, Lysobacter and Anaeroplasma,* while NOD-nursed mice had higher proportions of *Odoribacter, Bacteroides, Prevotella, Clostridium, Stenotrophomonas,* and *Akkermansia* (Figure 5A). At 32 weeks of age, NOR-nursed mice had higher proportions of *Prevotella*, *Parabacteroides, Christenella* and *Anaeroplasma,* while NOD-nursed mice had higher proportions of Odoribacter, *Allobaculum* and *Clostridium* (Figure 5B). Note that [*Prevotella*], in both Figure 5A and 5B, is a provisional taxanomical assignment by Greengenes of OTUs different from canonical *Prevotella*, and is not found in NCBI.

Changes of the intestinal microbiota due to age differ by nursing mother

Because samples also clustered by age, differences in intestinal microbiota by age were studied. The largest differences between the two time points were visible at the

phylum level, so statistical tests were run at the phylum and genus levels. Because samples clustered by nursing mother, analyses were stratified by nursing mother. From 4 weeks to 32 weeks of age, NOR-nursed mice had increases in the phylum Tenericutes and decreases in the phyla Bacteroidetes and Firmicutes and in the genus *Candidatus Arthomitus*. During the same period, NOD-nursed mice had increases in the phyla Firmicutes and Tenericutes and in the genus *Coprococcus*, and decreases in the phyla Bacteroidetes and Verrucomicrobia and in the genera *Bacterioides, Candidatus Arthomitus*, *Clostridium*, and *Stenotrophomonas*.

Discussion

Grouping based on bacterial sequences from fecal pellets of NOR and NOD mice revealed that the nursing mother, not the birth mother, dictates the composition of intestinal microbiota. Not only are these different groupings present at weaning (~4wks) but remain throughout the lifetime of the mice (32wks). Altering the microbiota to improve disease incidence has been previously achieved in the NOD mouse through the gavage of cecal contents from male mice into female mice. In these studies the gavage of cecal contents was not started until weaning and changes in microbiota were measured at 14 and 34wks of age(27). These results do not indicate the effects of inducing a microbiota shift earlier in life or to what extent the initial cecal gavage at 6 weeks is altering the microbiota. Ubeda, *et al*. have previously shown that the composition of microbiota in mice is largely determined by maternal transfer to pups regardless of genetic background, although they did not look at the cross-fostering of two different strains (18). Cross-fostering experiments after Cesarean section indicate the importance of the ability of the nursing mother to dictate the microbial composition over birth mothers (28). However, the ability to cause a long lasting microbial shift after vaginal birth is not addressed.

We successfully cross-fostered mice by switching the pups within 48hrs of birth. However, one potential difficulty of cross-fostering experiments is the rejection of fostered pups by nursing mothers. We did not encounter this problem, but there are potential alternative methods to deceive mothers into accepting fostered pups that may be used. One such alternative is to cover the pups with urine from the nursing mother, giving the pups the scent of the nursing mother. A second alternative is to administer petroleum

jelly onto the noses of nursing mothers on a daily base to prevent them from smelling fostered pups. Both methods may be used in place of the simple pup to mother switch described in the materials and methods section of this paper.

When focusing on T1D, cross-fostering brings to light some interesting trends. In children with T1D the Firmicutes to Bacteroidetes ratio was significantly lower when compared to age matched healthy children (29). During cross-fostering, mice nursed by a NOD mother and mice nursed by NOR mothers had a similar ratio of Firmicutes and Bacteroidetes as that seen in diabetic compared to healthy control in both mice and humans, with a higher ratio of Firmicutes to Bacteroidetes seen in mice nursed by a diabetic resistant NOR mother (5). The role of the other bacterial phylum (Verrucomicrobia, TM7, and Tenericutes) that are significantly different between mice nursed by NOD or NOR mothers in the context of T1D is not yet known. Looking beyond the phylum level, mice nursed by a NOD mother were positive for C*lostridium*, while mice nursed by a NOR mother were negative. This data mirrors what is seen in human subjects in which *Clostridium* levels are higher in diabetic children compared to healthy children (29).

In our analyses, there were two taxonomical groups identified as Prevotella: one having the canonical Prevotella sequence, and one having a non-canonical sequence provisionally assigned to Prevotella by Greengenes (indicated as [Prevotella]). In mice nursed by NOR mothers there is a higher proportion of [Prevotella] at 4 weeks and 32 weeks when compared to mice nursed by NOD mothers. However, NOD fostered mice have a higher proportion of canonical Prevotella at 4 weeks. Differences in Prevotella proportions in microbial communities are seen in children, with healthy children having a

higher ration of Prevotella compared to children with T1D (29). Interestingly Prevotella is found at a higher level in colorectal cancer patients and patients with Crohn's disease (CD) compared to healthy controls, indicating that the role of Prevotella varies between diseases (30, 31). Through cross-fostering we have removed some diabetogenic bacteria, while adding bacteria that are associated with diabetic resistance into NOD mice by having them nursed by NOR mothers. We also observed that in mice nursed by NOD or NOR mothers that there was a decrease in *Candidatus Arthromitus* between 4 and 32 weeks. *Candidatus Arthromitus* was identified as a segmented filamentous bacterium (SFB) in the gut of arthropods and has recently been shown to play an important role in the maturation of the immune system in the mouse gut (32, 33). This warrants additional investigation because Kriegel *et al.* showed the presence of SFB in female NOD mice, correlated with a decrease in the incidence of T1D (34). It may be possible in the future to use cross-fostering to manipulate levels of *Candidatus Arthromitus* to alter disease incidence. Further research will be required to determine if higher frequencies of Firmicutes, TM7, Tenericutes, and Verrucomicrobia, as seen in mice nursed by a NOR mother, can have an effect on disease pathogenesis. The incidence of diabetes will have to be monitored in future cross-fostering experiments to determine if phylum changes that occur as a result of cross-fostering are beneficial. This data indicates that cross-fostering appears to be a viable method to switch microbiota between strains and potentially protect mice from specific diseases; however it is also clear that bacteria that are protective from one disease may be promoting a different disease. So, simply cross-fostering to shift microbiota to what is thought to be a healthy state most likely will not protect from all disease and could possible increase susceptibility to other diseases.

For future microbiota studies, cross-fostering starting at birth appears to be a viable method to induce a shift in microbiota that remains for the entire lifespan of the mice. We are confident that this method could be used for other strains of mice and is not exclusive to the NOD and NOR strains of mice.

Acknowledgements

The authors would like to Mason Harris for animal husbandry and cross-fostering. This study was supported in part by the NIH grants P01 DK071176 and UL1TR00165, the Juvenile Diabetes Research Foundation Grant #36-2008-930, the Crohn's and Colitis Foundation of American Grant #26971, and the University of Alabama at Birmingham Digestive Diseases Research Development Center grant P30 DK064400. The following are acknowledged for their support of the Microbiome Resource at the University of Alabama at Birmingham: School of Medicine, Comprehensive Cancer Center (P30AR050948), Center for AIDS Research (5P30AI027767) and Center for Clinical Translational Science (UL1TR000165). Aspects of this project were conducted in biomedical research space that was constructed with funds supported in part by NIH grant C06RR020136. The Institutional Care and Use Committee and the Institutional Review Board of the University of Alabama at Birmingham approved all experiments. Sequencing was performed by the Heflin Center Genomics Core which is partially supported by the Cancer Center Core grant number P30 CA013148. The Institutional Care and Use Committee and the Institutional Review Board of the University of Alabama at Birmingham approved all experiments.

References

1. Ahern PP, Faith JJ, Gordon JI. Mining the Human Gut Microbiota for Effector Strains that Shape the Immune System. Immunity. 2014;40(6):815-23. doi: 10.1016/j.immuni.2014.05.012. PubMed PMID: 24950201.
2. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nature reviews Immunology. 2009;9(5):313-23. doi: 10.1038/nri2515. PubMed PMID: 19343057.
3. Sekirov I, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. Physiological reviews. 2010;90(3):859-904. doi: 10.1152/physrev.00045.2009. PubMed PMID: 20664075.
4. Wolf KJ, Daft JG, Tanner SM, Hartmann R, Khafipour E, Lorenz RG. Consumption of Acidic Water Alters the Gut Microbiome and Decreases the Risk of Diabetes in NOD Mice. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2014;62(4):237-50. doi: 10.1369/0022155413519650. PubMed PMID: 24453191; PubMed Central PMCID: PMC3966285.
5. Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, Drew JC, Ilonen J, Knip M, Hyoty H, Veijola R, Simell T, Simell O, Neu J, Wasserfall CH, Schatz D, Atkinson MA, Triplett EW. Toward defining the autoimmune microbiome for type 1 diabetes. The ISME journal. 2011;5(1):82-91. doi: 10.1038/ismej.2010.92. PubMed PMID: 20613793; PubMed Central PMCID: PMC3105672.
6. Suzuki T. Immune-Deficient Animals in Biomedical Research1987:112-6.
7. Duck LW, Walter MR, Novak J, Kelly D, Tomasi M, Cong Y, Elson CO. Isolation of flagellated bacteria implicated in Crohn's disease. Inflammatory bowel diseases. 2007;13(10):1191-201. doi: 10.1002/ibd.20237. PubMed PMID: 17712838.
8. Manichanh C, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. Nature reviews Gastroenterology & hepatology. 2012;9(10):599-608. doi: 10.1038/nrgastro.2012.152. PubMed PMID: 22907164.
9. Hildebrand F, Nguyen TL, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P, Liston A, Raes J. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome biology. 2013;14(1):R4. doi: 10.1186/gb-2013-14-1-r4. PubMed PMID: 23347395; PubMed Central PMCID: PMC4053703.
10. Garrett WS, Lord GM, Punit S, Lugo-Villarino G, Mazmanian SK, Ito S, Glickman JN, Glimcher LH. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. Cell. 2007;131(1):33-45. doi: 10.1016/j.cell.2007.08.017. PubMed PMID: 17923086; PubMed Central PMCID: PMC2169385.
11. Peng J, Narasimhan S, Marchesi JR, Benson A, Wong FS, Wen L. Long term effect of gut microbiota transfer on diabetes development. Journal of autoimmunity. 2014. doi: 10.1016/j.jaut.2014.03.005. PubMed PMID: 24767831.
12. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505(7484):559-63. doi: 10.1038/nature12820. PubMed PMID: 24336217; PubMed Central PMCID: PMC3957428.

13. Hansen CH, Krych L, Nielsen DS, Vogensen FK, Hansen LH, Sorensen SJ, Buschard K, Hansen AK. Early life treatment with vancomycin propagates Akkermansia muciniphila and reduces diabetes incidence in the NOD mouse. Diabetologia. 2012;55(8):2285-94. doi: 10.1007/s00125-012-2564-7. PubMed PMID: 22572803.
14. Schwartz RF, Neu J, Schatz D, Atkinson MA, Wasserfall C. Comment on: Brugman S et al. (2006) Antibiotic treatment partially protects against type 1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes? Diabetologia 49:2105-2108. Diabetologia. 2007;50(1):220-1. doi: 10.1007/s00125-006-0526-7. PubMed PMID: 17119915.
15. Goodrich JK, Di Rienzi SC, Poole AC, Koren O, Walters WA, Caporaso JG, Knight R, Ley RE. Conducting a Microbiome Study. Cell. 2014;158(2):250-62. doi: 10.1016/j.cell.2014.06.037. PubMed PMID: 25036628.
16. Thoene-Reineke C, Fischer A, Friese C, Briesemeister D, Gobel UB, Kammertoens T, Bereswill S, Heimesaat MM. Composition of intestinal microbiota in immune-deficient mice kept in three different housing conditions. PloS one. 2014;9(11):e113406. doi: 10.1371/journal.pone.0113406. PubMed PMID: 25401702; PubMed Central PMCID: PMC4234647.
17. Rogers GB, Kozlowska J, Keeble J, Metcalfe K, Fao M, Dowd SE, Mason AJ, McGuckin MA, Bruce KD. Functional divergence in gastrointestinal microbiota in physically-separated genetically identical mice. Scientific reports. 2014;4:5437. doi: 10.1038/srep05437. PubMed PMID: 24961643; PubMed Central PMCID: PMC4069701.
18. Ubeda C, Lipuma L, Gobourne A, Viale A, Leiner I, Equinda M, Khanin R, Pamer EG. Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. J Exp Med. 2012;209(8):1445-56. doi: 10.1084/jem.20120504. PubMed PMID: 22826298; PubMed Central PMCID: PMC3409501.
19. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, Hu C, Wong FS, Szot GL, Bluestone JA, Gordon JI, Chervonsky AV. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature. 2008;455(7216):1109-13. doi: 10.1038/nature07336. PubMed PMID: 18806780; PubMed Central PMCID: PMC2574766.
20. Seekatz AM, Aas J, Gessert CE, Rubin TA, Saman DM, Bakken JS, Young VB. Recovery of the Gut Microbiome following Fecal Microbiota Transplantation. mBio. 2014;5(3). doi: 10.1128/mBio.00893-14. PubMed PMID: 24939885.
21. Borody TJ, Paramsothy S, Agrawal G. Fecal microbiota transplantation: indications, methods, evidence, and future directions. Current gastroenterology reports. 2013;15(8):337. doi: 10.1007/s11894-013-0337-1. PubMed PMID: 23852569; PubMed Central PMCID: PMC3742951.
22. Smits LP, Bouter KE, de Vos WM, Borody TJ, Nieuwdorp M. Therapeutic potential of fecal microbiota transplantation. Gastroenterology. 2013;145(5):946-53. doi: 10.1053/j.gastro.2013.08.058. PubMed PMID: 24018052.
23. Kumar R, Eipers P, Little RB, Crowley M, Crossman DK, Lefkowitz EJ, Morrow CD. Getting started with microbiome analysis: sample acquisition to bioinformatics. Current protocols in human genetics / editorial board, Jonathan L Haines [et al]. 2014;82:18 8 1- 8 29. doi: 10.1002/0471142905.hg1808s82. PubMed PMID: 25042718.

24. Lozupone CA, Hamady M, Kelley ST, Knight R. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Applied and environmental microbiology. 2007;73(5):1576-85. doi: 10.1128/AEM.01996-06. PubMed PMID: 17220268; PubMed Central PMCID: PMC1828774.
25. Navas-Molina JA, Peralta-Sanchez JM, Gonzalez A, McMurdie PJ, Vazquez-Baeza Y, Xu Z, Ursell LK, Lauber C, Zhou H, Song SJ, Huntley J, Ackermann GL, Berg-Lyons D, Holmes S, Caporaso JG, Knight R. Advancing our understanding of the human microbiome using QIIME. Methods in enzymology. 2013;531:371-444. doi: 10.1016/B978-0-12-407863-5.00019-8. PubMed PMID: 24060131.
26. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460-1. doi: 10.1093/bioinformatics/btq461. PubMed PMID: 20709691.
27. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, von Bergen M, McCoy KD, Macpherson AJ, Danska JS. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science. 2013;339(6123):1084-8. doi: 10.1126/science.1233521. PubMed PMID: 23328391.
28. Hansen CH, Andersen LS, Krych L, Metzdorff SB, Hasselby JP, Skov S, Nielsen DS, Buschard K, Hansen LH, Hansen AK. Mode of Delivery Shapes Gut Colonization Pattern and Modulates Regulatory Immunity in Mice. Journal of immunology. 2014. doi: 10.4049/jimmunol.1400085. PubMed PMID: 24951818.
29. Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, Queipo-Ortuno MI. Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. BMC medicine. 2013;11:46. doi: 10.1186/1741-7015-11-46. PubMed PMID: 23433344; PubMed Central PMCID: PMC3621820.
30. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. PloS one. 2012;7(6):e39743. doi: 10.1371/journal.pone.0039743. PubMed PMID: 22761885; PubMed Central PMCID: PMC3386193.
31. Willing BP, Dicksved J, Halfvarson J, Andersson AF, Lucio M, Zheng Z, Jarnerot G, Tysk C, Jansson JK, Engstrand L. A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. Gastroenterology. 2010;139(6):1844-54 e1. doi: 10.1053/j.gastro.2010.08.049. PubMed PMID: 20816835.
32. Bolotin A, de Wouters T, Schnupf P, Bouchier C, Loux V, Rhimi M, Jamet A, Dervyn R, Boudebbouze S, Blottiere HM, Sorokin A, Snel J, Cerf-Bensussan N, Gaboriau-Routhiau V, van de Guchte M, Maguin E. Genome Sequence of "Candidatus Arthromitus" sp. Strain SFB-Mouse-NL, a Commensal Bacterium with a Key Role in Postnatal Maturation of Gut Immune Functions. Genome announcements. 2014;2(4). doi: 10.1128/genomeA.00705-14. PubMed PMID: 25035333; PubMed Central PMCID: PMC4102870.
33. Thompson CL, Vier R, Mikaelyan A, Wienemann T, Brune A. 'Candidatus Arthromitus' revised: segmented filamentous bacteria in arthropod guts are members of Lachnospiraceae. Environmental microbiology. 2012;14(6):1454-65. doi: 10.1111/j.1462-2920.2012.02731.x. PubMed PMID: 22436008.

34. Kriegel MA, Sefik E, Hill JA, Wu HJ, Benoist C, Mathis D. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(28):11548-53. doi: 10.1073/pnas.1108924108. PubMed PMID: 21709219; PubMed Central PMCID: PMC3136249.

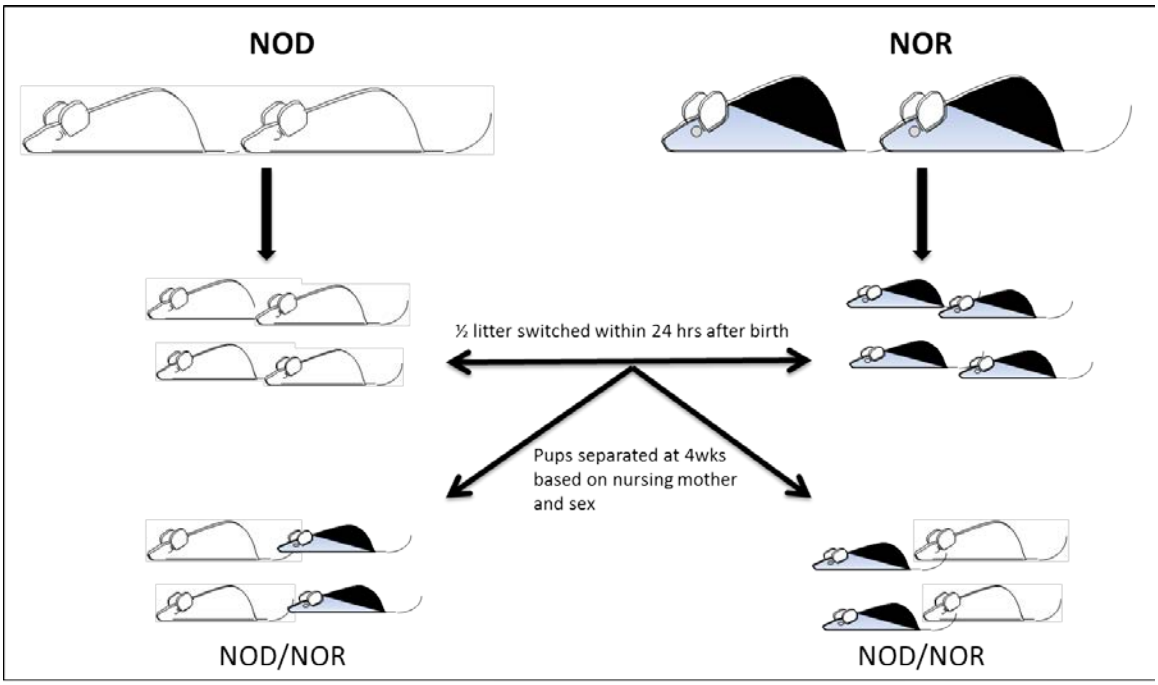


Figure 1. Experimental design of cross-fostering between mice of opposite strains. Breeding pairs of NOD and NOR mice are set up simultaneously. Pups that are born within 48 hours of each other to their respective parent are switched to a nursing mother of a different strain. Only half of the litters are switched, leaving half of each original litter with their birth mother. At weaning pups are separated based on sex and nursing mother, not on birth mother. Resulting cages will then contain mice of the same sex, but of mixed strains.

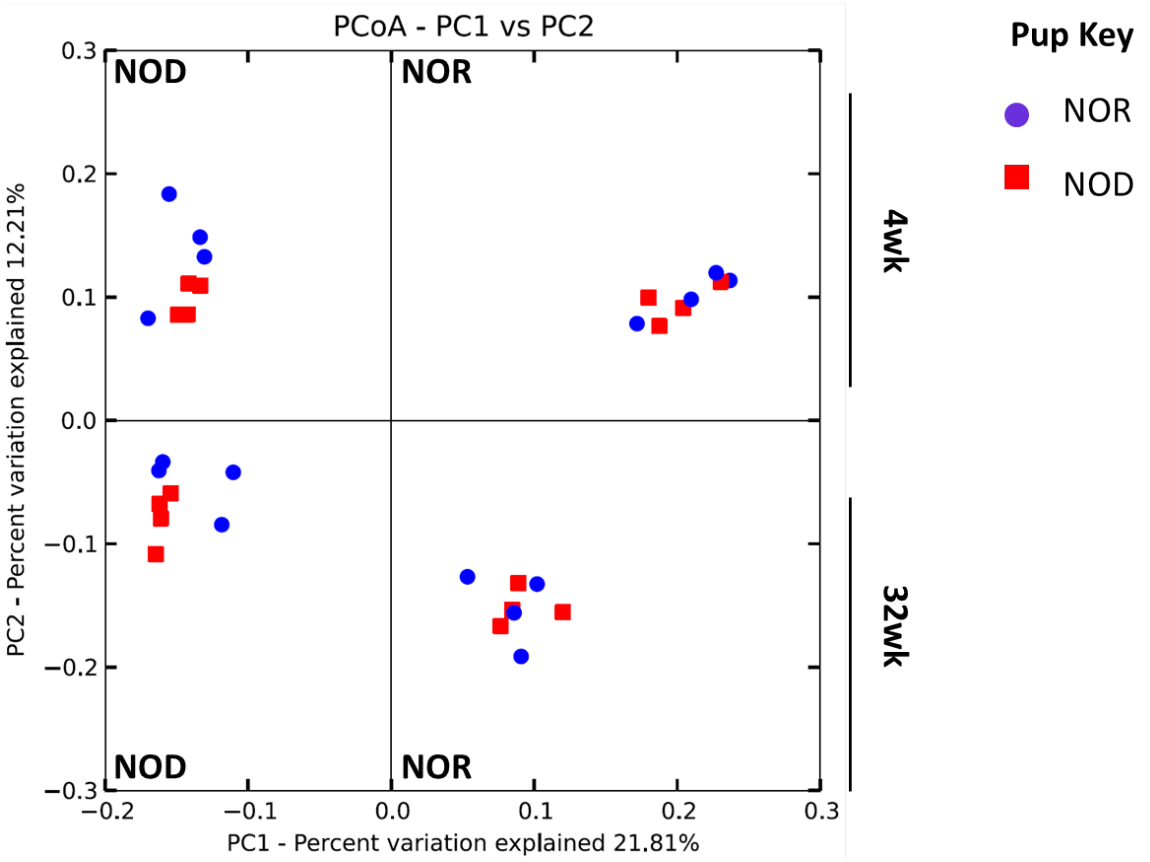


Figure 2. Grouping of fecal bacterial groups from mice nursed by a NOD or NOR mother, each quadrant is labeled with NOD or NOR, indicating nursing mother. PCoA plots were generated from bacterial DNA that was isolated from mouse fecal material and the V4 segment of the 16S rRNA gene was amplified from fecal pellets from mice nursed by NOD and NOR mothers. Group clustering represents a difference in β-diversity between mice nursed by NOD or NOR mothers. Significant differences ($p<0.05$) in beta diversity were calculated using compare_categories.py using the PERMANOVA test.

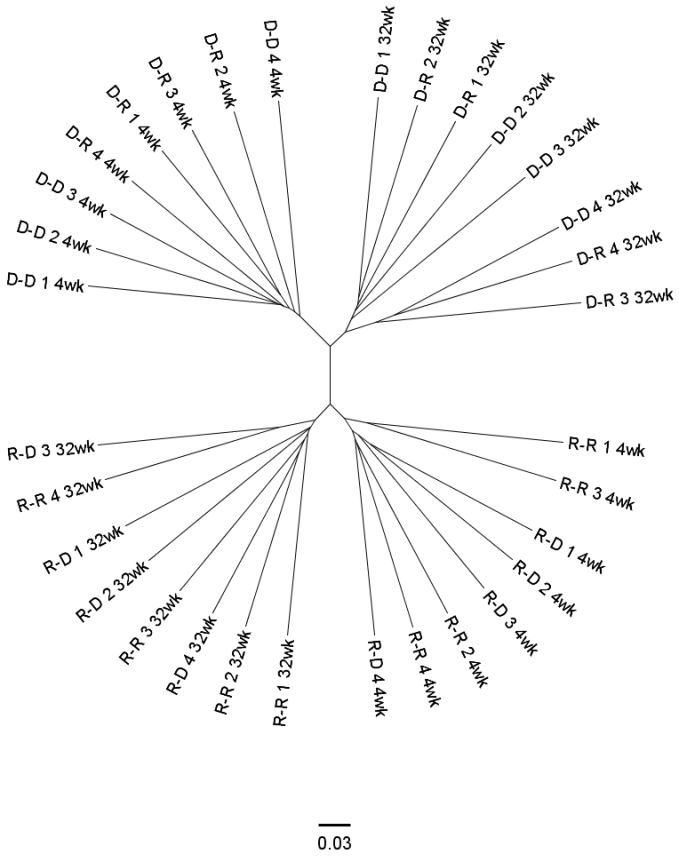
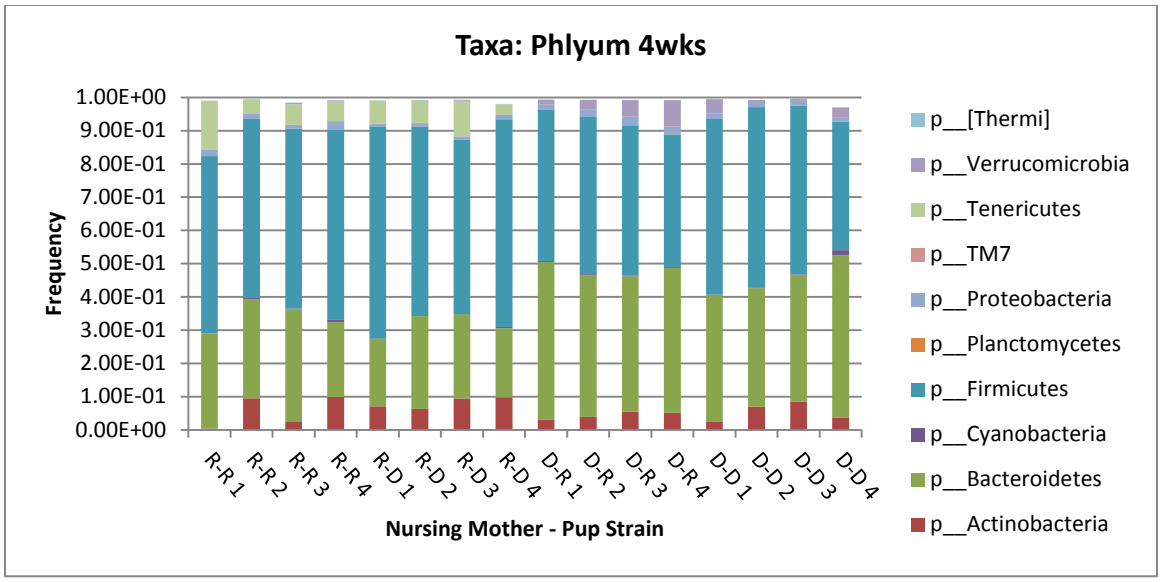


Figure 3. Phylogenetic tree of fecal bacteria from nice nursed by a NOD or NOR mother. Banding similarity analysis of the samples from NOD and NOR mice reveal that the highest degree of similarity exist between mice that were nursed by the same mother regardless of pup strain. A high degree of similarity also exists between groups based on age with groupings at 4 and 32 weeks. The mice are identified with the following naming convention: Nursing Mother-Pup Strain (where R = NOR and D = NOD).

A)



B)

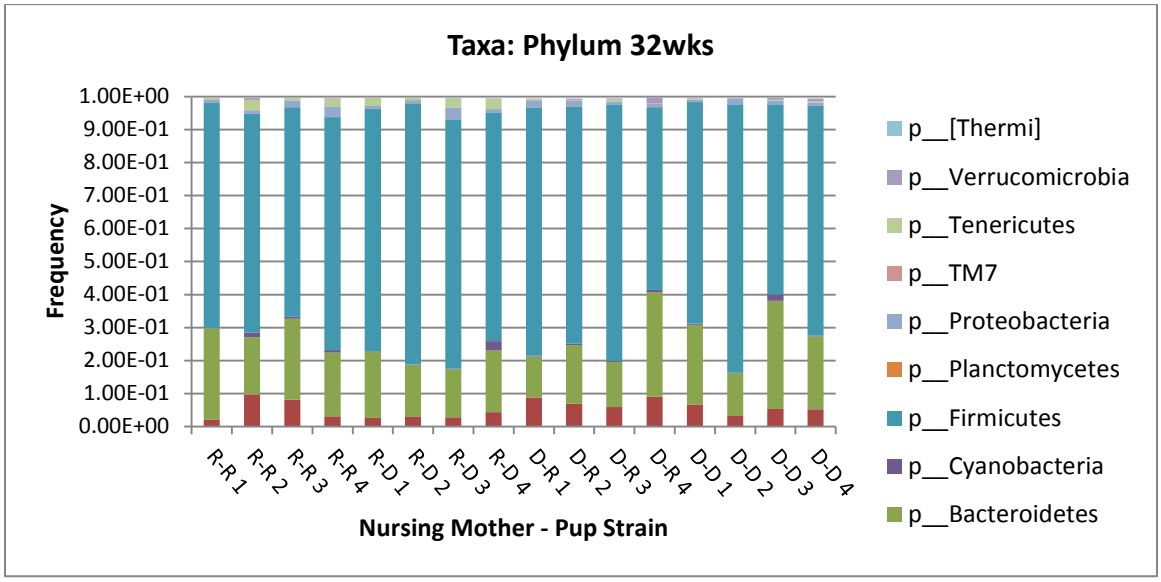
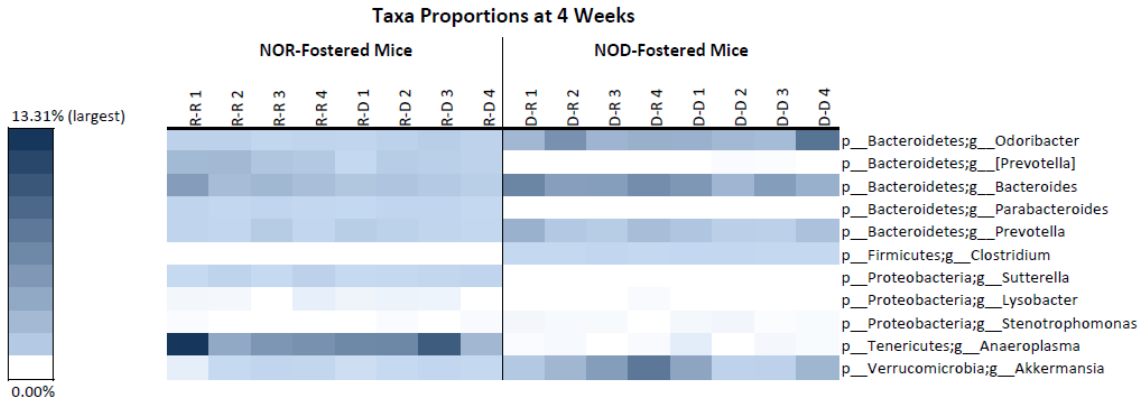


Figure 4. Phyla proportions in mice fostered by NOR and NOD mothers. These are stacked bar charts showing the cumulative proportions of bacterial phyla from NOR and NOD fostered mice. The mice are identified on the x axis with the following naming convention: Nursing Mother-Pup Strain (where R = NOR and D = NOD). **A:** Proportions at 4 weeks of age. **B:** Proportions at 32 weeks of age.

A)



B)

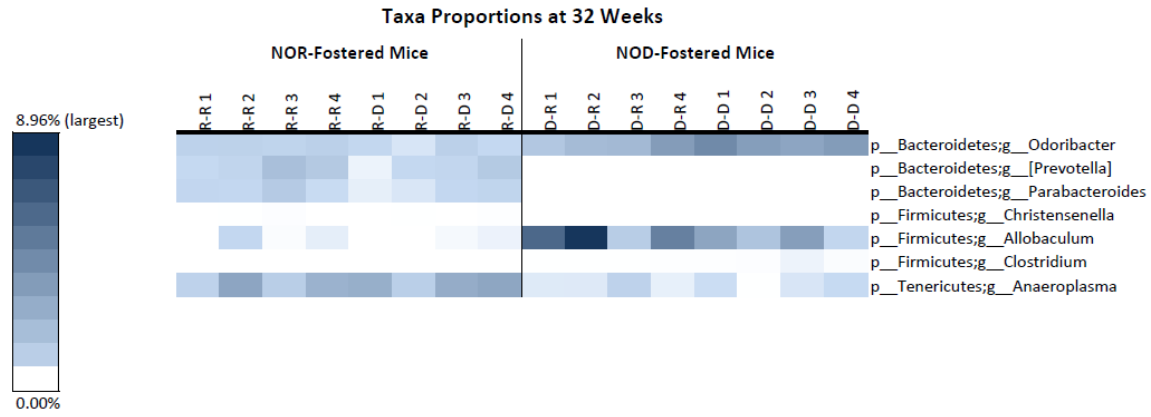


Figure 5. Significant differences in genera proportions between NOR and NOD fostered pups. This is a heatmap showing bacterial genera with statistically significant differences in proportion ($p<0.05$ after FDR correction) between NOR and NOD fostered mice at 4 weeks of age. Color shading is based on the proportion of each genera within fecal samples from each mouse, with the darkest color being the highest observed proportion (by time point) and white being zero (i.e. not present). Mouse ID is shown above each column with the following naming convention: Nursing Mother-Pup Strain (where R = NOR and D = NOD). **A:** Differences at 4 weeks of age. **B:** Differences at 32 weeks of age.

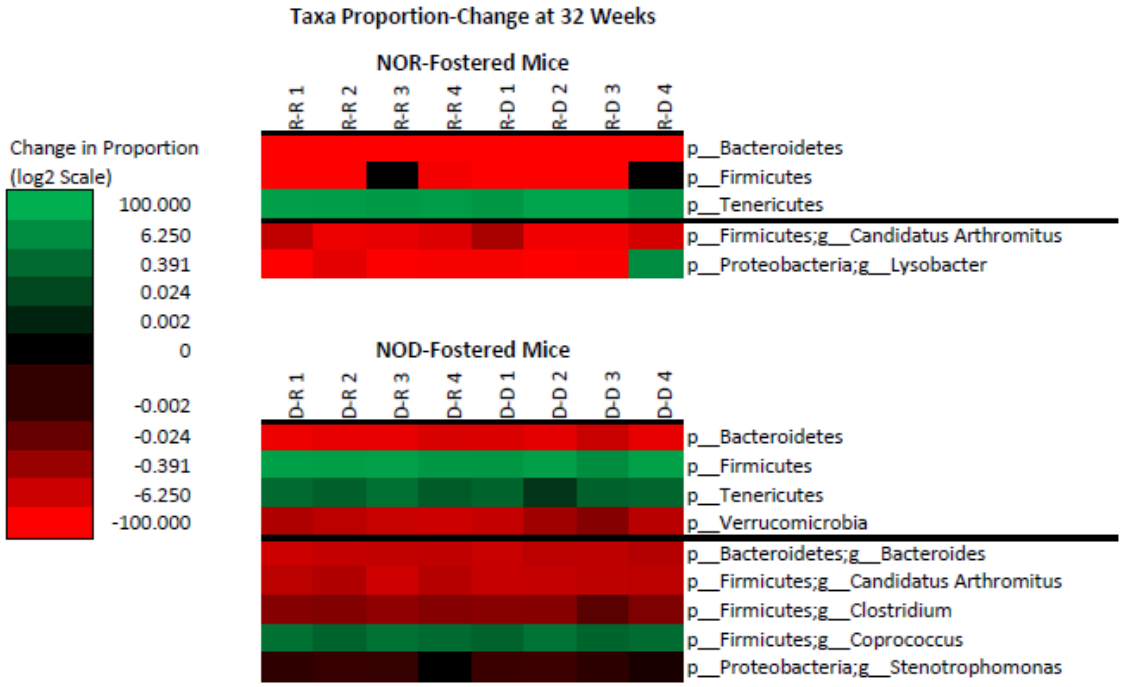


Figure 6. Significant changes in phyla and genera proportions from 4 weeks to 32 weeks of age in NOR and NOD fostered pups. This is a heatmap showing statistically significant changes in the proportions of phyla and genera ($p<0.05$ after FDR correction) from 4 weeks of age to 32 weeks. NOR and NOD fostered pups are shown separately. Color shading is based on the absolute change in taxa proportion on a log-2 scale, with green indicating increases in proportion, red indicating decreases in proportion, and black indicating no change. Mouse ID is shown above each column with the following naming convention: Nursing Mother-Pup Strain (where R = NOR and D = NOD).

REDUCED EXPRESSION OF ANTIMICROBIAL PEPTIDES AND MUCINS IN THE NOD MOUSE PRECEDES THE COLONIZATION OF A DIABETES PROMOTING MICROBIOTA

JOSEPH G DAFT, CASEY MORROW, ROBIN G LORENZ

In preparation for *Microbiome*

Format adapted for dissertation

Abstract:

Non-obese diabetic (NOD) mice have abnormalities in their gastrointestinal (GI) ecosystem that have been proposed as potential mechanisms for type 1 diabetes (T1D) pathogenesis. Controls typically used for comparison to NOD mice often do not have these abnormalities and therefore are not the most relevant control models. Despite a high level of genetic similarity and a permeable intestinal barrier like NOD mice, non-obese diabetic resistant (NOR) mice do not develop disease. Our data shows that NOR mice have increased expression of anti-microbial peptides (AMPs) and mucins in their distal ileum compared to NOD mice. This increased expression of AMPs and mucins is associated with increased antimicrobial activity using a mucus kill assay, as measured by *E. coli* colony counts. Our data also shows that after intestinal injury is induced using lethal irradiation, NOR mice respond with a significant increase in regenerative crypts when compared to NOD mice. This data suggests that NOR mice have an inherent ability to control the colonization of their GI ecosystem and recover at a higher degree from intestinal insults compared to NOD mice. These differences provide insight into disease pathogenesis and represent potential therapeutic targets.

Introduction

The role of commensal microbiota in disease has recently become a major research focus. Two questions are at the forefront of this field: 1) What role is commensal microbiota playing in controlling homeostasis of the immune system, and 2) What factors lead to the differences in commensal microbiota between healthy individuals and individuals who are predisposed to disease. The majority of research has focused on the first question with little research addressing the causes of the baseline differences in commensal microbiota between healthy and predisposed individuals. Unfortunately, in the past many experiments have not taken into consideration the potential differences in the GI ecosystem between experimental mouse strains and control strains. This has resulted in wide variation in data obtained from different institutions. There is now a large volume of research pointing to the incredible importance of intestinal microbiota in shaping the adaptive immune system (1-6). This can now be coupled with reports showing that mice of the same strain housed in different cages within the same facility have up to a 30% variance in their microbiota (7-11). Therefore it is critical to determine what host factors are responsible for shaping each mouse strains' commensal microbiota.

The GI ecosystem consists of three components: the commensal microbiota, the GI epithelium, and the mucosal immune system (12). Two key regulators of bacteria in the GI tract are antimicrobial peptides (AMPs) and mucins. In mice, rats, and humans, AMPs, which can be thought of as natural antibiotics, are produced by intestinal epithelial cells and in humans AMPs can also be produced by neutrophils (13). These

AMPs are typically cationic and are thought to be attracted to negatively charged phospholipids on the surface of bacterial cells where they insert themselves, interrupting the cell membrane and inducing cell death (13). One main group of AMPs are the defensins, which are known as cryptidins in mice and rats (14, 15). A major class of defensins are α-defensins, which are primarily expressed in the small intestine by Paneth cells located in the small intestinal crypts (16, 17). Another important AMP is RegIIIγ, which is produced by epithelial enterocytes and Paneth cells. RegIIIγ targets gram-positive bacteria and is responsible for providing a physical barrier that separates the microbiota from the epithelial barrier (18). Mucins are primarily produced by goblet cells throughout the intestinal tract and are complex glycoproteins that protect mucosal epithelial cells through steric hindrance, forming a physicochemical barrier, or through specific mucin-bacteria interactions (6, 19). Together AMPs and mucins contribute to the maintenance of the microbial community. What is still unknown is whether AMPs and mucins are produced in response to microbiota or if their strain-associated baseline production is shaping the early bacterial colonization of the GI tract.

The role of AMPs and mucins in the regulation of the microbiota in type 1 diabetes (T1D) remains largely unknown. However, recent research has brought to light the importance of microbiota in T1D incidence in animal models and in humans. In the non-obese (NOD) mouse model and the bio-breeding diabetes prone (BBdp) rat, the presence or absence of specific bacterial strains can alter disease incidence (20-25). Data gathered from humans subjects indicates that healthy subjects have a higher percentage of butyrate-producing and mucin degrading bacteria in their GI tracts compared to subjects with T1D (26). In children that are at risk for developing T1D, there is an elevation in

Bacteroidetes levels and a decrease in Firmicutes in their GI tract compared to age matched healthy controls (27). Importantly the effects of the microbiota on the incidence of T1D can be seen in the transfer of cecal content between mice. By transferring male cecal content to female mice the incidence of diabetes in female mice is significantly reduced, indicating there is a protective property of male microbiota in this model, presumably through increased levels of testosterone (21).

To study the effects of AMPs and mucins on shaping the intestinal commensal microbiota and on the incidence of T1D we have utilized the non-obese diabetic resistant (NOR) mouse as a control to be paired with the NOD mouse (28). The NOR mouse shares a high level of genetic similarity to the NOD mouse, develops peri-insulitis and also like the NOD has a "leaky" gut as we will show later in this publication. Previous reports have shown that NOR mice have non-permeable intestinal barriers when measured at approximately 12 weeks of age (29, 30). However the permeability of the GI tract in NOR mice at other ages requires further investigation. The concept of the leaky gut contributing to disease pathogenesis in T1D is becoming more appreciated and may need to be an important component of any diabetic experimental control strain moving forward (29, 31, 32). Despite these similarities to NOD mice, NOR mice raised in the same facility and on the same diet as NOD mice have a different microbiota and do not develop T1D. We hypothesize that NOD mice are susceptible to diabetes because of this altered microbiota, and that the microbiota is determined by strain specific AMP and mucin expression.

Methods

Animals

NOD/ShiLtJt mice and NOR/LtJ mice originally obtained from Jackson Laboratory (Bar Harbor, Maine) were bred and maintained under specific pathogen-free (SPF) conditions. All animals were housed in Thoren Isolator ventilated racks (Hazelton, PA). All caging, bedding, and food were sterilized prior to use. Both NOD and NOR mice were put on acidified water within a pH range of 3-3.5. Water was acidified using 1N HCl. Mice were fed autoclaved NIH-31 rodent diet (Harlan Teklan, Madison, WI) ad libitum. The Institutional Care and Use Committee of the University of Alabama at Birmingham approved all experiments. A detailed list of our facility's SPF conditions can be accessed at http://main.uab.eud/sites/ComparativePathology/surveillance/.

Sample prep and sequencing

Fecal microbiome DNA libraries were isolated and sequenced as previously described (33). Briefly, microbial DNA was isolated from mouse fecal material and the V4 segment of the 16S rRNA gene was amplified, and the product was used to create a library for next generation sequencing. Sequencing was performed on an Illumina Miseq using a 251bp paired-end library. Sequencing was performed at the UAB Heflin Center for Genomic Sciences.

Sequence analysis

Microbiome sequence libraries were analyzed using the Quantitative Insight into Microbial Ecology (QIIME) suite version 1.7 (34, 35). For analysis we used a wrapper for QIIME called QWRAP. Analysis with QWRAP was performed as previously described (33). Prior to analysis with QWRAP, we merged the overlapping forward and

reverse reads using the fastq_mergepairs tool from the USEARCH package (36). Read pairs with more than 5% mismatches were discarded. Output for QWRAP includes taxa summary tables (frequency of all given taxa by sample, by taxanomic level), alpha diversity measurements, and distance matricies and principle coordinate plots for beta diversity.

Intestinal Permeability measured by presence of antibodies to luminal antigens

Whole blood was collected by cheek bleed at 5 and 9 weeks of age. Whole blood was spun down at 12,000 rcf for 10 minutes and serum was collected and placed in a -20^{o} C until needed. Immunlon 4 Assay plates (Fisher, Pittsburgh, PA) were coated with Goat anti-mouse Ig (Dynatech) to establish an IgG standard. Plates were also coated with 0.5 ug/ml of CBir1 flagellin (generously provided by Charles O. Elson III) to detect anti-CBir1 flagellin. CBir1 flagellin is from a commensal bacterium belonging to *Clostridium* XIVa cluster and has yet to be cultured. An IgG standard was generated by doing a log3 dilution of mouse IgG (SouthernBiotech, Birmingham AL) and was used to estimate the concentration of anti-CBir1 flagellin IgG. For detection of mouse IgG (SouthernBiotech), AP labeled goat anti-mouse IgG antibody was used along with pNPP (Sigma Aldrich, St. Louis, MO). The developing reaction was stopped by 3N NaOH and the plate was read at 405 nm.

Antimicrobial peptide and mucin expression (mRNA)

The distal ileum was removed and the lumen was flushed with 1xPBS and the placed overnight in RNA*later* ®(Ambion®, Carlsbad, CA) at 4^{0}C. Tissue was then homogenized in TRIZOL® reagent (Ambion®) to isolate RNA, which was then stored at -80^{o} C. Genomic DNA was removed from RNA samples using the Turbo DNA-free

protocol from Ambion®. cDNA was then synthesized from ileal RNA samples using the Roche (South San Francisco, CA) Transcriptor First Strand cDNA Synthesis Kit. RT-PCR was performed using primers from Applied Biosystems® and TaqMan® from Life Technologies (Applied Biosystems®). 18S ribosomal DNA was used to normalize to target genes. Crossing thresholds were used to determine statistical significance and determine the fold change in expression between the ileum of NOD and NOR mice.

Mucus Kill Assay

The mucus kill assay was modified from Meyer-Hoffert et. Al (37). Briefly ileums are removed, rinsed with 1xPBS, and opened exposing the lumen. Glass slides are then used to scrape the mucus layer off of the luminal side of the ileum. When the mucus layer has been removed, it is then placed into 200ul 1xPBS. The mucus/PBS is vortexted thoroughly until mucus is evenly dispersed. 100ul of *E. coli* with an ampicillin resistant gene grown to an AU of 0.6 – 0.7 read at OD 600 was added to the mucus/PBS and again vortexed. For the control 100ul of *E. coli* was added to 200ul PBS without mucus. The *E. coli* mucus culture along with the control was spun down at 1500 rpm for 15 minutes at room temperature and diluted (1/100000) in LB broth. 100ul of the final dilution is plated on LB agar plates with ampicillin and incubated at 37^{o}C. Colonies from the mucus *E. coli* mixture were counted the next day and compared back to the colony count of the control plate.

Antibiotic treatment of mice

Breeding pairs of NOD and NOR mice were set up and immediately put on an antibiotic cocktail that was delivered via their drinking water. Water was autoclaved and allowed to cool before the antibiotics were added (streptomycin sulfate (2.4mg/ml),

ampicillin (0.6mg/ml), metronidazole (0.6mg/ml) and vancomycin (0.3mg/ml)). Once the antibiotics have dissolved, the water is acidified with 1N HCl. Breeding pairs remain on antibiotic water during gestation and nursing. When pups reach two weeks of age they are sacrificed and their distal Ileum are collected to measure RNA expression. RNA expression is measured as mentioned in *Antimicrobial peptide and mucin expression*.

Cellular division within intestinal crypts

Proliferating/regenerating cells were identified based on 5-bromodeoxyuridine (BrdUrd) (Sigma) incorporation and detection by anti-BrdUrd (1/1000) as described by Cohn *et al*. (38). Animals were injected i.p. with BrdUrd (120mg/kg) 90 min prior to sacrifice. Tissue was fixed at sacrifice using cold Bouin's Fixative (Fisher). Tissue was then processed and analyzed as previously described by Staley *et al.* (39). BrdUrd positive cells were counted in 10 consecutive crypts of the ileac crypts to determine the number of dividing cells per crypt.

Irradiation

Male mice were subjected to lethal (9 Gy) doses of X-ray irradiation at 5 or 10 weeks of age in the X-RAD 320 irradiator (Precision X-Ray Inc., N. Branford, CT), in accordance with previous studies evaluating cellular regeneration and repair. Crypt regeneration was quantified in animals 3.5 days after irradiation as described previously (40). Distal ileum was harvested and fixed in Bouin's solution (Fisher, Pittsburgh, PA). Tissue was then cut into 5 mm segments and embedded longitudinally in paraffin to allow for cross-sectional analysis. Regenerative crypts were defined as crypts having 5 or more BrdUrd positive cells. An average of 6 intestinal cross-sections were analyzed per ileum.

Cross-fostering

Breeding pairs of NOD and NOR mice were simultaneously set up when individual mice reached approximately 6 weeks of age. Pups born to NOD and NOR breeding pairs within 24 hours of each other were used for cross-fostering. After both NOD and NOR litters had been born, half of each litter was removed and put with the mother of the opposite strain. Litters then contained pups born to that nursing mother and pups of the opposite NOD or NOR strain (Fig. 8). Fostered pups were marked daily with a sharpie on the back of the neck until their ears were able to be clipped (approximately 7 days) for identification purposes. The pups were nursed by their birth mother or nursing mother until weaning. At weaning pups were separated based on sex, but not strain.

Diabetes Incidence

Mice were monitored weekly by measuring urine glucose using Diastix® (Bayer) starting at 8 weeks of age. Following a positive urine test, a blood glucose test was performed the next day using the OneTouch® Blood Glucose Meter (Greenwood Village, CO). Diabetes was defined as a positive urine test followed by blood glucose test of 200mg/dl or greater. Once mice were defined as diabetic they were sacrificed and removed from the study.

Serum IgA levels

Whole blood was collected by cheek bleed at weaning (~4wks) and was spun down at 12,000 rcf for 10 minutes and serum was collected and placed in a -20^{o} C until needed. Immunlon 4 Assay plates (Fisher) were coated with Goat anti-mouse Ig (Dynatech) to establish an IgA standard and detect serum IgA. An IgA standard was generated by doing a log3 dilution of mouse IgA (SouthernBiotech) and was used to

determine total IgA in serum (1/1500 dilution). For detection, a goat anti-mouse IgG (SouthernBiotech), AP labeled antibody was used along with pNPP (Sigma Aldrich). The developing reaction was stopped by 3N NaOH and the plate was read at 405 nm.

Results

NOD and NOR mice have a different microbiota prior to diabetes onset in NOD mice:

At weaning (4wks) the bacterial populations within the GI system of diabetes susceptible NOD mice differ from diabetes resistant NOR mice. At 4 weeks NOD mice have a higher frequency of Bacteroidetes, Deferribacteres, and Verrucomicrobia in their GI systems when compared age matched NOR after amplification of 16s PCR (Fig. 1). Conversely, NOR mice at 4 weeks have a higher percentage of Firmicutes and TM7 in their GI system compared to age matched NOD mice (Table 1). These results support previously published data, that show children at risk for T1D also have an increase in Bacteroidetes and a decrease in Firmicutes compared to age matched healthy subjects (27). The difference in commensal microbiota between NOD and NOR mice can then be measured by antibodies in serum due the permeable intestinal barrier of both strains of mice.

NOD and NOR mice generate antibodies to cecal antigens

Permeable intestinal barriers are associated with T1D and many control models used for studying the disease do not have permeable barriers. We propose that the NOR mouse is a viable control and that the permeability of the NOR GI barrier must be measured. Cecal serum probes using serum from a female diabetic NOD mouse and an age matched NOR mouse, revealed a unique band at approximately 100kD (Fig. 2). This band was identified as containing multiple flagellin antigens including CBir1 flagellin. Antibodies (IgG) to CBir1 flagellin, a bacterial antigen from the gut lumen, were detected in the serum of female NOD and NOR mice at 5 and 9 weeks (Fig. 3) indicating that

these mice have permeable guts, which is also seen in BB rats and humans predisposed to T1D (32, 41). Other common mouse models used for diabetic research do not typically have permeable guts, as demonstrated by C57BL/6 mice which do not have detectable levels of CBir1 flagellin antibodies in their serum at theses time points (data not shown). It has previously been shown that both the FVB and C3H/HeJ, commonly used control strains for intestinal inflammatory disease models, lack intestinal permeability as measured by serum reactivity to the luminal flagellin antigens, CBir1 and Fla-X (42). For this reason the NOR mouse, which has a permeable intestinal barrier, is a unique diabetic control that we feel has been underutilized.

Ileal expression of AMPs and Mucins differ between NOD and NOR mice

At 2 weeks and 5 weeks mRNA expression of AMPs and mucins differs between NOD and NOR mice. At 2 weeks NOD mice have a higher expression of the AMPs, Defcr-4, Defcr-r, and RegIIIγ (Fig. 4A). By 5 weeks a significant difference in Defr-4, Defcr-r, and RegIIIγ no longer exists between NOD and NOR mice (Fig. 4B). However, at 5 weeks a significant increase in MMP7 expression was measured in the Ileum of NOD mice. MMP7 is responsible for cleaving defensins, a class of AMPs, from their pre to pro form, so although AMP expression is not changed at 5 weeks between NOD and NOR mice, activity may be affected. Mucin expression is higher in the NOD mice at 5 weeks of age compared to NOR mice, with a significant increase in mRNA expression of Muc2 in NOD mice. These variations in AMP and mucin expression are likely related to the difference in microbiota between the two strains of mice. Changes in AMP and mucin expression between 2 and 5 weeks reveals a very dynamic and changing environment in the GI ecosystem of NOD and NOR mice. What remains unknown is

whether the microbiota is leading to the difference in AMP and mucin expression or if the difference in AMP and mucin expression is a result of the already present microbiota.

NOR mice have a greater ability to regulate bacteria in their ileum compared to NOD mice.

To determine if the difference seen in commensal microbiota between NOD and NOR mice is a result of an alteration in ability to control bacterial growth a mucus kill assay was performed. When *E. coli* is cultured with intestinal mucus containing mucins and AMPs, when compared to age and gender matched NOD mice, a significant reduction in colony forming units (cfu's) is seen at 5 weeks in the NOR samples (Fig. 5A). At 10 weeks there is no difference in cfu's when samples are cultured with mucus from NOD and NOR mice (Fig. 5B). This indicates that earlier in life (5wks) NOR mice have a better ability to regulate bacterial numbers in their ileum compared to NOD. However as mice age this difference in ability to control bacterial numbers is no longer significantly different between the two strains of mice.

NOR mice dictate the colonization of microbiota through AMP and mucin expression.

Changes in AMP and mucin expression along with a decreased functional ability to control bacteria exist in NOD, when compared to NOR mice; however, it is unknown as to whether AMP and mucin expression is dictating bacterial colonization or if colonization is determining AMP and mucin expression. Using antibiotics to simulate germfree conditions, mRNA expression of AMPs and mucins in the ileum of 2 week old NOD mice was significantly reduced compared to NOR mice (Fig. 6). In the absence of bacteria there is a reduction in expression of MMP7, RegIIIγ, and Muc2 in NOD mice

when using the expression of these genes in NOR mice as a base line. The reduction in expression of AMPs and mucins in germfree NOD mice indicates that at basal levels, NOR mice do not rely as heavily on bacterial load to produce these peptides. This may be leading to colonization by a different microbiota between the two strains of mice later in life (Fig. 1). The observed differences in AMP and mucin expression between animals that are susceptible to disease and those that are not, needs to be further explored to determine if this observation is true in other models of T1D. AMP and mucin expression early in life may serve as a marker for susceptibility to developing T1D.

Cells within the crypts of the Ileum are more actively dividing in NOR mice when compared to NOD mice

To assess if alterations in the intestinal microbiota between NOD and NOR mice leads to a difference in response to intestinal injury and thus a potential mechanism for disease pathogenesis, cell division within intestinal crypts was measured. BrdUrd incorporation studies show a greater of number of dividing cells within the ileal crypts of NOR mice at both 2 weeks and 10 weeks when compared to age matched NOD mice (Fig. 7). The number of dividing cells within the ileal crypts increased in both strains at 10 weeks when compared back to their respective strains at 2 weeks. After intestinal injury is induced by irradiation, a significantly higher number of regenerative crypts were seen in NOR mice when compared to age matched NOD mice (Fig. 8). A higher number of regenerative crypts serves as a marker of an increased ability to heal after an insult and this epithelial response to injury appears to be stunted in NOD mice perhaps leaving them with a more permeable epithelial barrier and therefore, more susceptible to exposure to luminal antigens contributing to disease pathogenesis.

Cross-fostering alters diabetes incidence in NOD mice

We have previously shown that a distinct difference in commensal microbiota exists after cross-fostering, with colonization by a diabetic resistant microbiota in NOD pups nursed by a NOR mother. This allowed us to determine the effect on diabetes incidence after colonization by a NOR microbiota in NOD mice (Fig. 9). Cross-fostering in male NOD mice is protective, with no development of diabetes in NOD male mice nursed by a NOR mother compared to approximately 80% disease incidence in male NOD mice nursed by a NOD mother (Fig. 10A). Interestingly it has been shown that transfer of microbiota from male NOD mice, which have a higher resistance to disease development than do female mice, can lower disease incidence (21). The high incidence of diabetes in the male mice nursed by a NOD mother is possibly due to our small sample size or to the composition of the microbiota in our NOD colonies. However, this same protection is not seen in female NOD mice nursed by a NOR mother (Fig. 10B), future studies are needed to determine the gender difference in disease incidence. However the sample size of male NOD mice nursed by a NOR mother is n=10 and supports a protective role for cross-fostering in male mice.

Cross-fostering alters serum IgA levels in NOD mice

Cross-fostering leads to an alteration to the commensal microbiota in mice that are nursed by a NOD or NOR mother. IgA is an important regulator of microbiota, so alterations to the microbiota may lead ot alteration in IgA. Serum IgA levels are significantly higher in female mice nursed by a NOR mother compared to mice nursed by a NOD mother (Figure 11B). Interestingly although cross-fostering lowers diabetes incidence in male

mice nursed by a NOR mother, it does not have a significant effect on IgA levels of male mice nursed by a NOR mother (11A).

Discussion

Differences in the microbiota and alterations in intestinal homeostasis in healthy individuals, diseased individuals, and individual predisposed to disease have now been reported in multiple autoimmune diseases including diabetes, inflammatory bowel disease, and even rheumatoid arthritis (2-6, 43). For years the study of autoimmune diseases such as those mentioned above used controls that did not address potential variations in the microbiota and intestinal homeostasis between the diseased state and the control animal or subject. Animal studies of T1D have typically used control animals such as C57BL/6 and BALB/c mice, which do not have altered intestinal homeostasis in addition to differing microbiota and may not be the best controls for studying T1D (29, 44, 45). To properly address the importance of the alterations of intestinal permeability and intestinal homeostasis in the NOD mouse we have proposed the use of the NOR mouse. The NOR mouse comes from a similar genetic background as the NOD mouse, sharing the common T1D polymorphism in the MHCII complex ($H2g^7$) and other diabetes associated loci (46). The NOR mouse also has alterations in intestinal permeability as seen in NOD mice. These similarities between NOD and NOR make the NOR mouse a prime candidate for studying the role of microbiota and intestinal homeostasis in disease development. Due to their similarities, this also allows for the exploration of difference between the two mouse strains and how they may protect or promote disease.

Our data indicates that NOR mice, like NOD mice, have a permeable intestinal barrier. In fact when measuring serum antibodies (IgG) to luminal antigen (CBir1 flagellin) we see an increased response in age matched NOR mice when compared to

NOD mice. Many reports point to a permeable gut being pathogenic, but this may not be the case as shown by the NOR mouse which does not develop diabetes. NOR mice have previously been shown to have an exaggerated immune response, when compared to NOD mice, MOG35-55 immunization; however, the autoimmune response was associated eith a reduce severity of clinical disease (47). This reveals that intestinal permeability alone is not the sole determinant of disease. Whether the higher number of dividing cells within the ileal crypts having a correlation with protection from T1D will hold in other animals models and humans remains unknown and requires further investigation.

The importance of microbiota is well established in the NOD mouse, where the transfer of microbiota from male NOD mice, which have lower incidence of diabetes, into female mice significantly lowers the incidence of diabetes in the receiving mice (21). In animal models and human subjects it is well established that there is a distinct difference in the microbiota of healthy subjects compared to diseased subjects. Multiple groups are investigating how microbiota can shape the immune response (8, 9, 11, 25, 27, 48). However, it is still not known what leads to differential bacterial colonization. Because NOD and NOR mice share many genetic similarities they are good candidates to investigate what is leading to these differences in bacterial colonization. Pathogenic bacteria are regulated by multiple factors, including mucins and AMPs (16, 49-52). Our data shows that in the presence of bacteria the expression level of AMPs and mucins varies between both strains of mice. This observation coupled with an established difference in the microbiota between these two strains reveals two possibilities: 1) colonization by bacteria induces AMP and mucin expression or 2) AMP and mucin

expression determines what bacteria are colonizing the gut. Through the use of antibiotics we established the equivalent of a germ free model and determined that in the absence of bacteria NOD mice have a significantly lower expression of MMP7, RegIIIγ and Muc3 compared to NOR mice. This illustrates that NOR mice appear to have a greater ability to control what bacteria are colonizing their GI system compared to NOD mice. Vaishnava et al. reports the importance of RegIIIγ in promoting a physical barrier that separates the microbiota from the epithelial surface (18). MMP7 has been shown to not only be important for controlling enteric pathogens through activation of defensins, but has also shown to be important for clearing away damaged tissue before epithelial repair begins (53). Infection with *Trichinella spiralis* in mice indicates that expression of Muc3 is an essential component to the innate response (54). Alteration in expression of any of these peptides (RegIIIγ, MMP7, Muc3) could therefore have a significant effect on disease pathogenesis. The expression level of AMPs and mucins in NOR mice appears to be conducive to have a healthy microbiota, while expression levels in NOD mice allows the colonization of diabetogenic promoting bacteria. The possibility may exist that AMP and mucin expression profiles could indicate disease susceptibility. Gene expression of AMPs and mucins alone are not enough to determine if they are having an effect on disease outcome. A mucus kill assay showed that at an early age, mucus, which contains AMPs and mucins, from NOR mice is better able to regulate bacterial growth (*E.* coli) than mucus from NOD mice. Further investigation of the role of AMPs and mucins is required to better understand their role in maintaining homeostasis and preventing disease in both animal models and humans.

Current research is focused on altering and controlling the microbiota to prevent disease (22, 23, 55, 56). There are multiple methods that are employed to restructure or introduce a healthy microbiota to a subject that is in a diseased state. In humans, fecal transplants have been used to successfully treat patients with *C. difficle* infections, but their use in other diseases requires further investigation (57-59). Methods currently used in animal models either require a large number of animals or repeated delivery of bacteria to try and shift the GI microbiota. One method that we've used to shift the microbiota is cross-fostering. By cross-fostering NOD pups with a NOR mother we have introduced the microbiota of NOR mice into NOD mice (Daft et al. Chapter 1). In male NOD mice, with which we have a larger sample size, the introduction of NOR microbiota provides a significant reduction in the incidence of diabetes. However, male mice fostered by a NOR mother do not have an elevated level of serum IgA compared to male mice nursed by a NOD mother. Although we have not seen a reduction in the incidence of diabetes in female NOD mice that were nursed by a NOR mother, we feel that a larger sample size will serve as a better indicator of the protective effects of the NOR microbiota. Interestingly in female NOD mice that are fostered by an NOR mother there is an elevation in serum IgA when compared to NOD mice nursed by an NOD mother. To further determine if levels of IgA are associated with disease incidence a larger sample size of female mice is needed, but based on data from male NOD mice the protective benefits of cross-fostering is not because of elevated levels of IgA.

In conclusion NOR mice have alterations in intestinal permeability as seen in NOD mice and share genetic abnormalities that are linked to T1D. These similarities make the NOR mouse an important control for studying T1D when using the NOD

mouse model. Comparison of these two models will provide important insight into what makes one strain susceptible and one strain protected from T1D.

References

1. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, Sitaraman SV, Knight R, Ley RE, Gewirtz AT. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science. 2010;328(5975):228-31. doi: 10.1126/science.1179721. PubMed PMID: 20203013.
2. Kamada N, Nunez G. Regulation of the Immune System by the Resident Intestinal Bacteria. Gastroenterology. 2014. doi: 10.1053/j.gastro.2014.01.060. PubMed PMID: 24503128.
3. Lee YK, Mazmanian SK. Has the microbiota played a critical role in the evolution of the adaptive immune system? Science. 2010;330(6012):1768-73. doi: 10.1126/science.1195568. PubMed PMID: 21205662; PubMed Central PMCID: PMC3159383.
4. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, Umesaki Y, Littman DR, Benoist C, Mathis D. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity. 2010;32(6):815-27. doi: 10.1016/j.immuni.2010.06.001. PubMed PMID: 20620945; PubMed Central PMCID: PMC2904693.
5. Conte MP, Schippa S, Zamboni I, Penta M, Chiarini F, Seganti L, Osborn J, Falconieri P, Borrelli O, Cucchiara S. Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease. Gut. 2006;55(12):1760-7. doi: 10.1136/gut.2005.078824. PubMed PMID: 16648155; PubMed Central PMCID: PMC1856471.
6. Vaarala O, Atkinson MA, Neu J. The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. Diabetes. 2008;57(10):2555-62. doi: 10.2337/db08-0331. PubMed PMID: 18820210; PubMed Central PMCID: PMC2551660.
7. Hildebrand F, Nguyen TL, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P, Liston A, Raes J. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome biology. 2013;14(1):R4. doi: 10.1186/gb-2013-14-1-r4. PubMed PMID: 23347395; PubMed Central PMCID: PMC4053703.
8. Ahern PP, Faith JJ, Gordon JI. Mining the Human Gut Microbiota for Effector Strains that Shape the Immune System. Immunity. 2014;40(6):815-23. doi: 10.1016/j.immuni.2014.05.012. PubMed PMID: 24950201.
9. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nature reviews Immunology. 2009;9(5):313-23. doi: 10.1038/nri2515. PubMed PMID: 19343057.
10. Round JL, O'Connell RM, Mazmanian SK. Coordination of tolerogenic immune responses by the commensal microbiota. Journal of autoimmunity. 2010;34(3):J220-5. doi: 10.1016/j.jaut.2009.11.007. PubMed PMID: 19963349; PubMed Central PMCID: PMC3155383.
11. Sekirov I, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. Physiological reviews. 2010;90(3):859-904. doi: 10.1152/physrev.00045.2009. PubMed PMID: 20664075.
12. McCracken V, Lorenz R. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. Cellular microbiology. 2001;3(1):1-11. PubMed PMID: 11207615.

13. Salzman NH, Underwood MA, Bevins CL. Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa. Seminars in immunology. 2007;19(2):70-83. doi: 10.1016/j.smim.2007.04.002. PubMed PMID: 17485224.
14. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nature immunology. 2000;1(2):113-8. doi: 10.1038/77783. PubMed PMID: 11248802.
15. Underwood MA, Kananurak A, Coursodon CF, Adkins-Reick CK, Chu H, Bennett SH, Wehkamp J, Castillo PA, Leonard BC, Tancredi DJ, Sherman MP, Dvorak B, Bevins CL. Bifidobacterium bifidum in a rat model of necrotizing enterocolitis: antimicrobial peptide and protein responses. Pediatric research. 2012;71(5):546-51. doi: 10.1038/pr.2012.11. PubMed PMID: 22322385; PubMed Central PMCID: PMC3619207.
16. Cunliffe RN. Alpha-defensins in the gastrointestinal tract. Molecular immunology. 2003;40(7):463-7. PubMed PMID: 14568393.
17. Kaiser V, Diamond G. Expression of mammalian defensin genes. Journal of leukocyte biology. 2000;68(6):779-84. PubMed PMID: 11129644.
18. Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, Ley R, Wakeland EK, Hooper LV. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science. 2011;334(6053):255-8. doi: 10.1126/science.1209791. PubMed PMID: 21998396; PubMed Central PMCID: PMC3321924.
19. Mack DR, Ahrne S, Hyde L, Wei S, Hollingsworth MA. Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. Gut. 2003;52(6):827-33. PubMed PMID: 12740338; PubMed Central PMCID: PMC1773687.
20. Valladares R, Sankar D, Li N, Williams E, Lai KK, Abdelgeliel AS, Gonzalez CF, Wasserfall CH, Larkin J, Schatz D, Atkinson MA, Triplett EW, Neu J, Lorca GL. Lactobacillus johnsonii N6.2 mitigates the development of type 1 diabetes in BB-DP rats. PloS one. 2010;5(5):e10507. doi: 10.1371/journal.pone.0010507. PubMed PMID: 20463897; PubMed Central PMCID: PMC2865539.
21. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, von Bergen M, McCoy KD, Macpherson AJ, Danska JS. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science. 2013;339(6123):1084-8. doi: 10.1126/science.1233521. PubMed PMID: 23328391.
22. Brugman S, Klatter FA, Visser JT, Wildeboer-Veloo AC, Harmsen HJ, Rozing J, Bos NA. Antibiotic treatment partially protects against type 1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes? Diabetologia. 2006;49(9):2105-8. doi: 10.1007/s00125-006-0334-0. PubMed PMID: 16816951.
23. Hansen CH, Krych L, Nielsen DS, Vogensen FK, Hansen LH, Sorensen SJ, Buschard K, Hansen AK. Early life treatment with vancomycin propagates Akkermansia muciniphila and reduces diabetes incidence in the NOD mouse. Diabetologia. 2012;55(8):2285-94. doi: 10.1007/s00125-012-2564-7. PubMed PMID: 22572803.

24. Sofi MH, Gudi RR, Karumuthil-Melethil S, Perez N, Johnson BM, Vasu C. pH of drinking water influences the composition of gut microbiome and type 1 diabetes incidence. Diabetes. 2013. doi: 10.2337/db13-0981. PubMed PMID: 24194504.
25. Wolf KJ, Daft JG, Tanner SM, Hartmann R, Khafipour E, Lorenz RG. Consumption of Acidic Water Alters the Gut Microbiome and Decreases the Risk of Diabetes in NOD Mice. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2014;62(4):237-50. doi: 10.1369/0022155413519650. PubMed PMID: 24453191; PubMed Central PMCID: PMC3966285.
26. Brown CT, Davis-Richardson AG, Giongo A, Gano KA, Crabb DB, Mukherjee N, Casella G, Drew JC, Ilonen J, Knip M, Hyoty H, Veijola R, Simell T, Simell O, Neu J, Wasserfall CH, Schatz D, Atkinson MA, Triplett EW. Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes. PloS one. 2011;6(10):e25792. doi: 10.1371/journal.pone.0025792. PubMed PMID: 22043294; PubMed Central PMCID: PMC3197175.
27. Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, Drew JC, Ilonen J, Knip M, Hyoty H, Veijola R, Simell T, Simell O, Neu J, Wasserfall CH, Schatz D, Atkinson MA, Triplett EW. Toward defining the autoimmune microbiome for type 1 diabetes. The ISME journal. 2011;5(1):82-91. doi: 10.1038/ismej.2010.92. PubMed PMID: 20613793; PubMed Central PMCID: PMC3105672.
28. Serreze DV, Chapman HD, Varnum DS, Gerling I, Leiter EH, Shultz LD. Initiation of autoimmune diabetes in NOD/Lt mice is MHC class I-dependent. Journal of immunology. 1997;158(8):3978-86. PubMed PMID: 9103469.
29. Lee AS, Gibson DL, Zhang Y, Sham HP, Vallance BA, Dutz JP. Gut barrier disruption by an enteric bacterial pathogen accelerates insulitis in NOD mice. Diabetologia. 2010;53(4):741-8. doi: 10.1007/s00125-009-1626-y. PubMed PMID: 20012858.
30. Hadjiyanni I, Li KK, Drucker DJ. Glucagon-like peptide-2 reduces intestinal permeability but does not modify the onset of type 1 diabetes in the nonobese diabetic mouse. Endocrinology. 2009;150(2):592-9. doi: 10.1210/en.2008-1228. PubMed PMID: 18845625.
31. Dahlquist G, Savilahti E, Landin-Olsson M. An increased level of antibodies to beta-lactoglobulin is a risk determinant for early-onset type 1 (insulin-dependent) diabetes mellitus independent of islet cell antibodies and early introduction of cow's milk. Diabetologia. 1992;35(10):980-4. PubMed PMID: 1451957.
32. Sapone A, de Magistris L, Pietzak M, Clemente MG, Tripathi A, Cucca F, Lampis R, Kryszak D, Carteni M, Generoso M, Iafusco D, Prisco F, Laghi F, Riegler G, Carratu R, Counts D, Fasano A. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. Diabetes. 2006;55(5):1443-9. PubMed PMID: 16644703.
33. Kumar R, Eipers P, Little RB, Crowley M, Crossman DK, Lefkowitz EJ, Morrow CD. Getting started with microbiome analysis: sample acquisition to bioinformatics. Current protocols in human genetics / editorial board, Jonathan L Haines [et al]. 2014;82:18 8 1- 8 29. doi: 10.1002/0471142905.hg1808s82. PubMed PMID: 25042718.
34. Lozupone CA, Hamady M, Kelley ST, Knight R. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Applied and environmental microbiology. 2007;73(5):1576-85. doi:

10.1128/AEM.01996-06. PubMed PMID: 17220268; PubMed Central PMCID: PMC1828774.
35. Navas-Molina JA, Peralta-Sanchez JM, Gonzalez A, McMurdie PJ, Vazquez-Baeza Y, Xu Z, Ursell LK, Lauber C, Zhou H, Song SJ, Huntley J, Ackermann GL, Berg-Lyons D, Holmes S, Caporaso JG, Knight R. Advancing our understanding of the human microbiome using QIIME. Methods in enzymology. 2013;531:371-444. doi: 10.1016/B978-0-12-407863-5.00019-8. PubMed PMID: 24060131.
36. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460-1. doi: 10.1093/bioinformatics/btq461. PubMed PMID: 20709691.
37. Meyer-Hoffert U, Hornef MW, Henriques-Normark B, Axelsson LG, Midtvedt T, Putsep K, Andersson M. Secreted enteric antimicrobial activity localises to the mucus surface layer. Gut. 2008;57(6):764-71. doi: 10.1136/gut.2007.141481. PubMed PMID: 18250125.
38. Cohn SM, Lieberman MW. The use of antibodies to 5-bromo-2'-deoxyuridine for the isolation of DNA sequences containing excision-repair sites. The Journal of biological chemistry. 1984;259(20):12456-62. PubMed PMID: 6490625.
39. Staley EM, Yarbrough VR, Schoeb TR, Daft JG, Tanner SM, Steverson D, Jr., Lorenz RG. Murine P-glycoprotein deficiency alters intestinal injury repair and blunts lipopolysaccharide-induced radioprotection. Radiation research. 2012;178(3):207-16. PubMed PMID: 22780103; PubMed Central PMCID: PMC3474324.
40. Riehl TE, Newberry RD, Lorenz RG, Stenson WF. TNFR1 mediates the radioprotective effects of lipopolysaccharide in the mouse intestine. American journal of physiology Gastrointestinal and liver physiology. 2004;286(1):G166-73. doi: 10.1152/ajpgi.00537.2002. PubMed PMID: 14525729.
41. Meddings JB, Jarand J, Urbanski SJ, Hardin J, Gall DG. Increased gastrointestinal permeability is an early lesion in the spontaneously diabetic BB rat. The American journal of physiology. 1999;276(4 Pt 1):G951-7. PubMed PMID: 10198339.
42. Lodes MJ, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, Fort M, Hershberg RM. Bacterial flagellin is a dominant antigen in Crohn disease. The Journal of clinical investigation. 2004;113(9):1296-306. doi: 10.1172/JCI20295. PubMed PMID: 15124021; PubMed Central PMCID: PMC398429.
43. Tiwana H, Wilson C, Walmsley RS, Wakefield AJ, Smith MS, Cox NL, Hudson MJ, Ebringer A. Antibody responses to gut bacteria in ankylosing spondylitis, rheumatoid arthritis, Crohn's disease and ulcerative colitis. Rheumatology international. 1997;17(1):11-6. PubMed PMID: 9194209.
44. Forbes EE, Groschwitz K, Abonia JP, Brandt EB, Cohen E, Blanchard C, Ahrens R, Seidu L, McKenzie A, Strait R, Finkelman FD, Foster PS, Matthaei KI, Rothenberg ME, Hogan SP. IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. The Journal of experimental medicine. 2008;205(4):897-913. doi: 10.1084/jem.20071046. PubMed PMID: 18378796; PubMed Central PMCID: PMC2292227.
45. O'Brien BA, Huang Y, Geng X, Dutz JP, Finegood DT. Phagocytosis of apoptotic cells by macrophages from NOD mice is reduced. Diabetes. 2002;51(8):2481-8. PubMed PMID: 12145161.

46. Prochazka M, Serreze DV, Frankel WN, Leiter EH. NOR/Lt mice: MHC-matched diabetes-resistant control strain for NOD mice. Diabetes. 1992;41(1):98-106. PubMed PMID: 1727742.
47. Mayo S, Quinn A. Altered susceptibility to EAE in congenic NOD mice: Altered processing of the encephalitogenic MOG35-55 peptide by NOR/LtJ mice. Clinical immunology. 2007;122(1):91-100. doi: 10.1016/j.clim.2006.08.001. PubMed PMID: 16990050.
48. Manichanh C, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. Nature reviews Gastroenterology & hepatology. 2012;9(10):599-608. doi: 10.1038/nrgastro.2012.152. PubMed PMID: 22907164.
49. Ganz T. Immunology. Versatile defensins. Science. 2002;298(5595):977-9. doi: 10.1126/science.1078708. PubMed PMID: 12411693.
50. Kim YS, Ho SB. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Current gastroenterology reports. 2010;12(5):319-30. doi: 10.1007/s11894-010-0131-2. PubMed PMID: 20703838; PubMed Central PMCID: PMC2933006.
51. Eckmann L. Defence molecules in intestinal innate immunity against bacterial infections. Current opinion in gastroenterology. 2005;21(2):147-51. PubMed PMID: 15711205.
52. Cunliffe RN, Mahida YR. Expression and regulation of antimicrobial peptides in the gastrointestinal tract. Journal of leukocyte biology. 2004;75(1):49-58. doi: 10.1189/jlb.0503249. PubMed PMID: 14525966.
53. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nature reviews Immunology. 2004;4(8):617-29. doi: 10.1038/nri1418. PubMed PMID: 15286728.
54. Shekels LL, Anway RE, Lin J, Kennedy MW, Garside P, Lawrence CE, Ho SB. Coordinated Muc2 and Muc3 mucin gene expression in Trichinella spiralis infection in wild-type and cytokine-deficient mice. Digestive diseases and sciences. 2001;46(8):1757-64. PubMed PMID: 11508679.
55. Vaarala O. Human intestinal microbiota and type 1 diabetes. Current diabetes reports. 2013;13(5):601-7. doi: 10.1007/s11892-013-0409-5. PubMed PMID: 23934614.
56. Kerman DH, Deshpande AR. Gut microbiota and inflammatory bowel disease: the role of antibiotics in disease management. Postgraduate medicine. 2014;126(4):7-19. doi: 10.3810/pgm.2014.07.2779. PubMed PMID: 25141239.
57. Seekatz AM, Aas J, Gessert CE, Rubin TA, Saman DM, Bakken JS, Young VB. Recovery of the Gut Microbiome following Fecal Microbiota Transplantation. mBio. 2014;5(3). doi: 10.1128/mBio.00893-14. PubMed PMID: 24939885.
58. Borody TJ, Paramsothy S, Agrawal G. Fecal microbiota transplantation: indications, methods, evidence, and future directions. Current gastroenterology reports. 2013;15(8):337. doi: 10.1007/s11894-013-0337-1. PubMed PMID: 23852569; PubMed Central PMCID: PMC3742951.
59. Smits LP, Bouter KE, de Vos WM, Borody TJ, Nieuwdorp M. Therapeutic potential of fecal microbiota transplantation. Gastroenterology. 2013;145(5):946-53. doi: 10.1053/j.gastro.2013.08.058. PubMed PMID: 24018052.

Table 1. Frequency of commensal microbiota at the phylum level in 4 week old female NOD and NOR mice.

Phylum	NOD Avg	NOD Stdev	NOR Avg	NOR Stdev	P value
Actinobacteria	5.7E-04	2.8E-04	7.8E-03	1.4E-02	0.29
Bacteroidetes	5.6E-01	8.9E-02	1.3E-01	1.7E-01	$p < 0.01$
Chloroflexi	5.4E-07	7.4E-07	0.0E+00	0.0E+00	0.14
Cyanobacteria	3.8E-04	4.2E-04	1.9E-05	2.3E-05	0.85
Deferribacteres	1.4E-05	3.2E-06	8.4E-06	3.3E-06	$p < 0.05$
Firmicutes	2.8E-01	7.8E-02	7.8E-01	1.8E-01	$p < 0.001$
Fusobacteria	3.8E-06	2.2E-06	9.0E-06	7.2E-06	0.16
Gemmatimonadetes	3.7E-07	8.2E-07	6.9E-07	9.5E-07	0.58
Proteobacteria	2.3E-02	3.6E-02	2.0E-03	2.3E-03	0.23
TM7	0.0E+00	0.0E+00	3.7E-05	4.3E-05	0.09
Tenericutes	7.1E-04	8.9E-04	2.6E-03	4.0E-03	0.34
Verrucomicrobia	6.4E-02	4.5E-02	2.1E-03	4.3E-03	$p < 0.05$

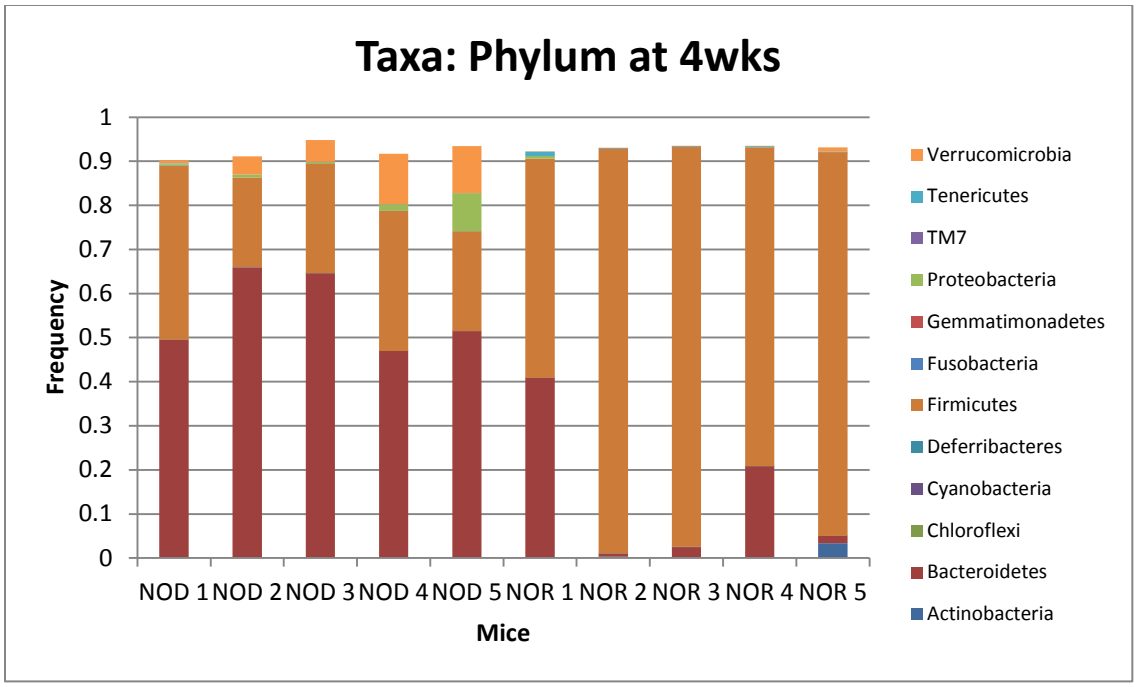


Figure 1. Phylogenetic analysis of bacteria from fecal pellets of 4wks female NOD (n=5) and NOR (n=5) mice. NOD mice had a significant increase in frequency of Bacteroidetes, Deferribacteres, and Verrucomicrobia when compared to NOR mice. A significant decrease in the frequency of Firmicutes and TM7 was measured when comparing NOD mice to NOR mice.

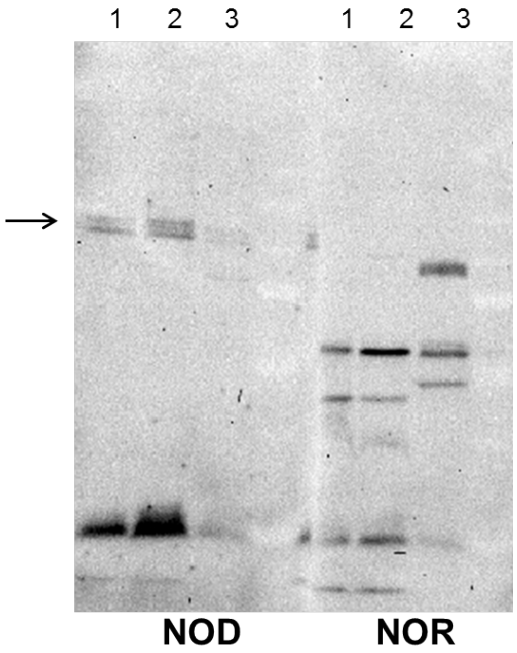


Figure 2. Cecal probe using serum from a female diabetic NOD mouse (25wks) and age matched NOR mouse. Lanes 1-3, contained cecal protein from 30 week male NOD mice (1-2) and 12 week old male NOD mice (3). A band was identified at approximately 100 kD in samples probed with NOD serum that was not seen in samples probed with NOR serum.

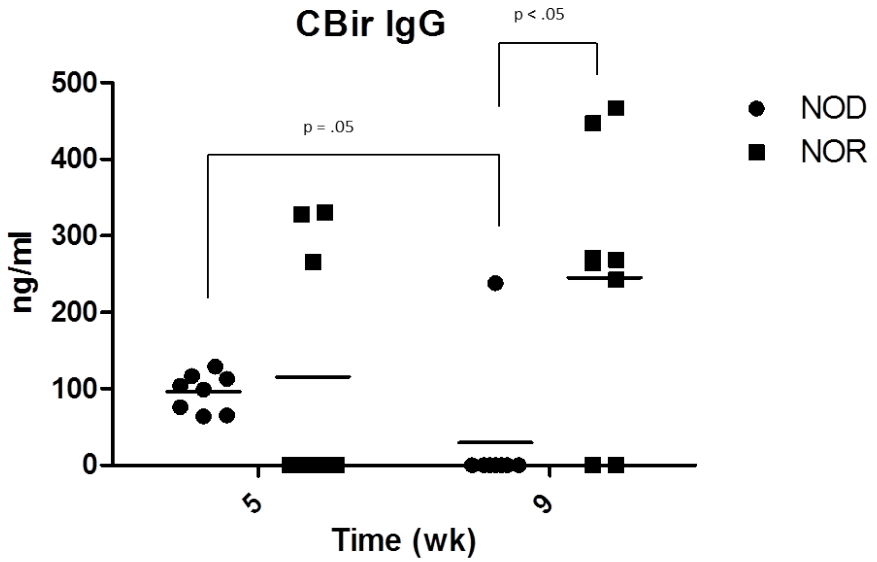
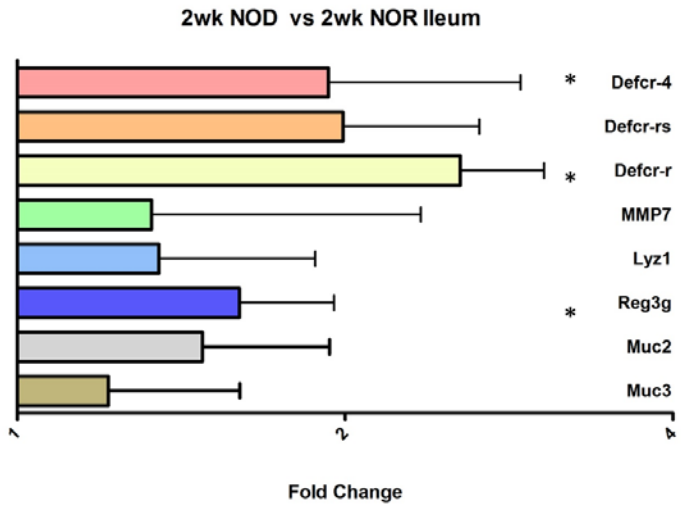


Figure 3. Anti-CBir1 flagellin (IgG) levels measured in serum collected from female NOD and NOR mice at 5 weeks and 9 weeks. Anti-CBir1 flagellin levels were measured by ELISA using the serum from female NOD (n=8) and NOR (n=7) mice. A significant increase ($p < 0.05$) in anti-CBir1 flagellin was measured in NOD and NOR mice at 9 weeks. A significant decrease ($p=0.05$) in anti-CBir1 flagellin was measured between NOD mice at 5 to 9 weeks.

A)



B)

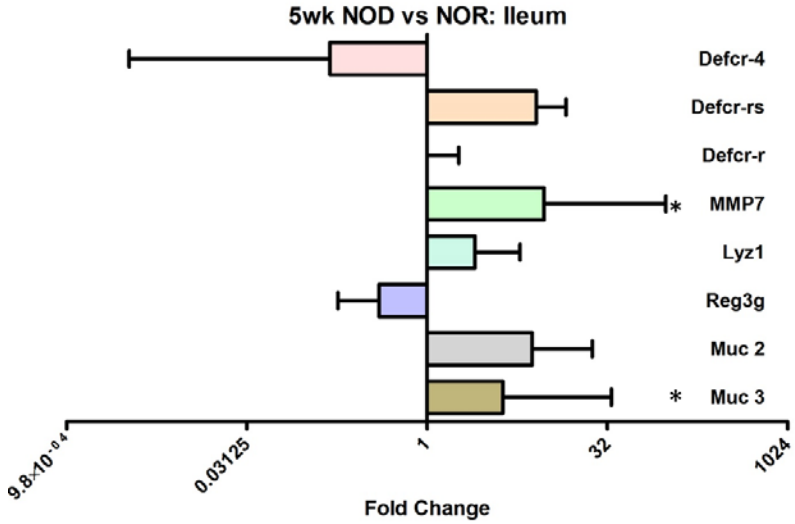
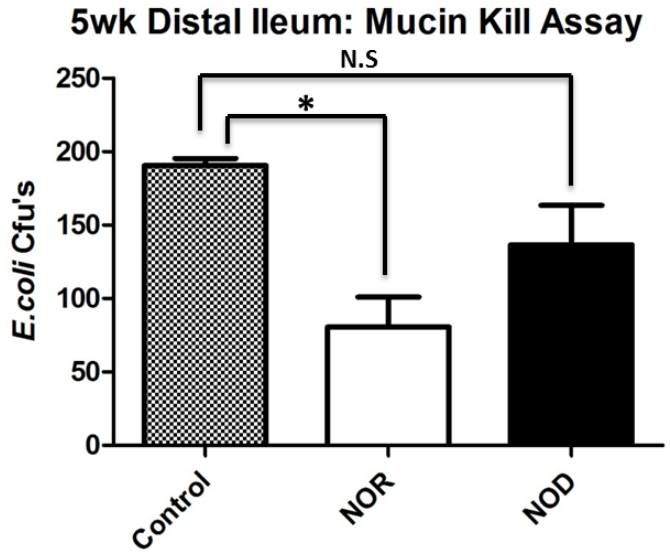


Figure 4. Expression (mRNA) of antimicrobial peptides (AMPs), mucins and matrix metalloproteinase-7 (MMP7) was measured from Ileal tissue by RT-PCR. Expression was reported as fold change in NOD (N=5) mice, using expression in NOR (n=7) mice as a baseline. A significant increase in expression of mRNA was measured in MMP7 ($p=0.05$) and Muc2 ($p<0.05$).

A)



B)

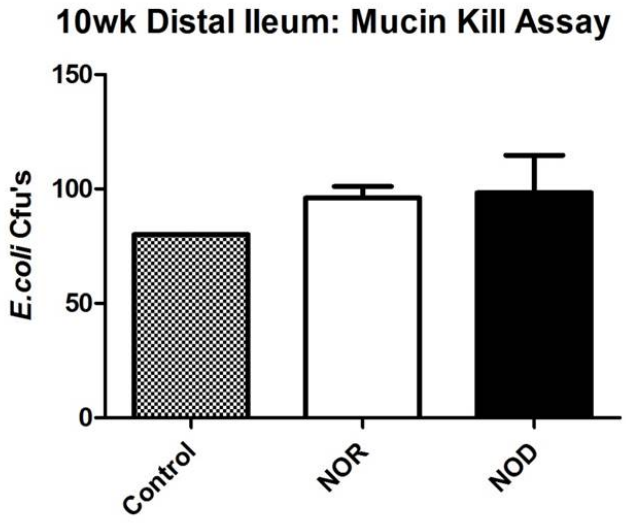


Figure 5. At 5wks (A) *E. coli* colony counts are significantly ($p<0.001$)reduced after being cultured with mucus from NOR (n=7) mice compared back to colonies that were cultured in the absence of mucus. This significant reduction in colony number is not seen when comparing culture with mucus from NOD (n=6) mice back to control. Colony counts are not significantly reduced when cultured in the presence of mucus from the ileum NOD or NOR mice at 10 weeks (B).

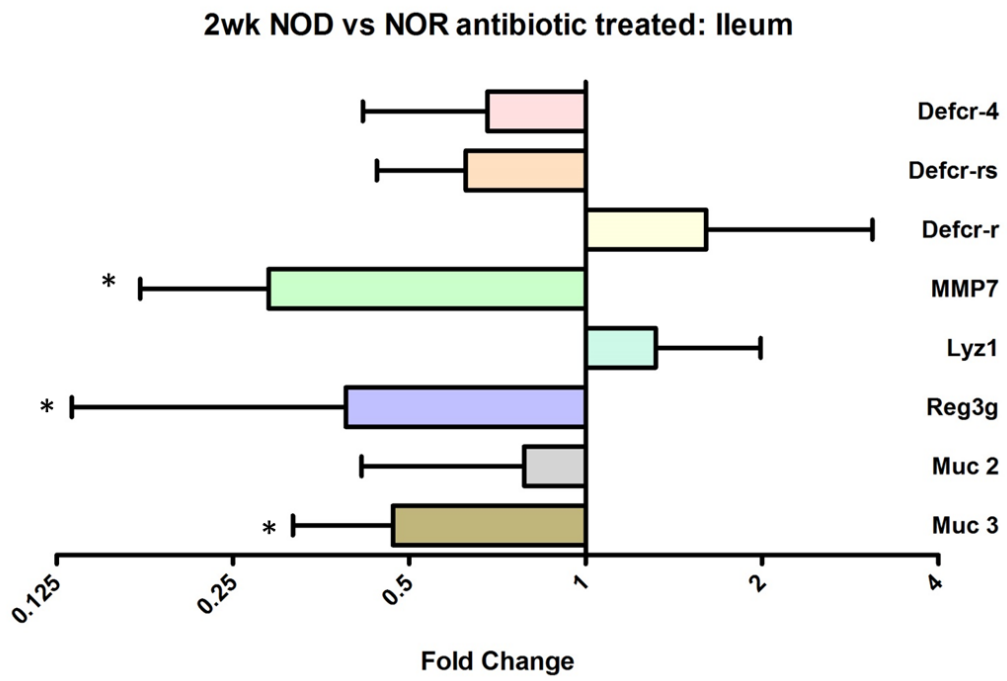


Figure 6. Expression (mRNA) of AMPs, mucins and MMP7 was measured from ileal tissue by RT-PCR in mice that had been treated with an antibiotic cocktail. Expression was reported as a fold change in NOD (n=8) mice, using expression in NOR (n=8) as a baseline. A significant reduction in MMP7 ($p<0.005$) and RegIIIγ ($p=0.05$) was measured along with trending decrease in Muc3 ($p=0.0586$).

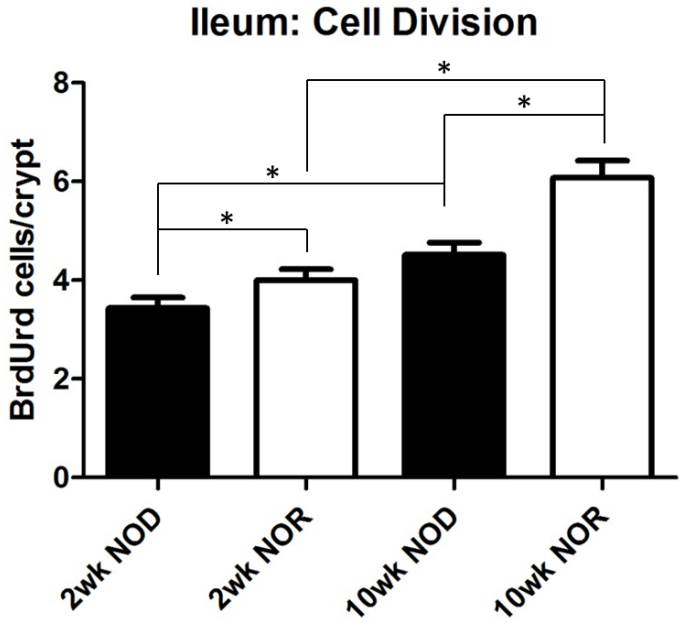


Figure 7. BrdUrd positive cells were counted in 10 consecutive crypts in the distal Ileum of each mouse to determine the average number of dividing cells per crypt. There was a significant ($p<0.05$) increase in the number of dividing cells in NOR mice at both 2wks (n=4) and 10wks (n=4) compared to NOD mice at 2wks (n=3) and 10wks (n=5) mice. In both strains the number of dividing cells significantly ($p<0.05$) increased within each individual strain from 2wks to 10wks.

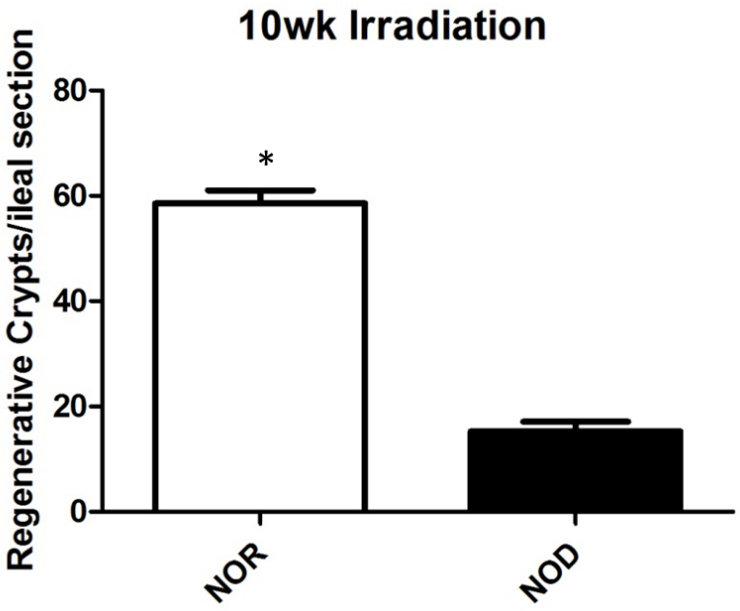


Figure 8. NOD (n=3) and NOR (n=4) mice were given a lethal dose of irradiation (9 Gray) at 10 weeks and regenerative crypts were identified using BrdUrd. Positive crypts were identified as crypts containing 5 or more BrdUrd positive cells. The increase in regenerative crypts in NOR mice was significant ($p<.01$) when compared to NOD mice.

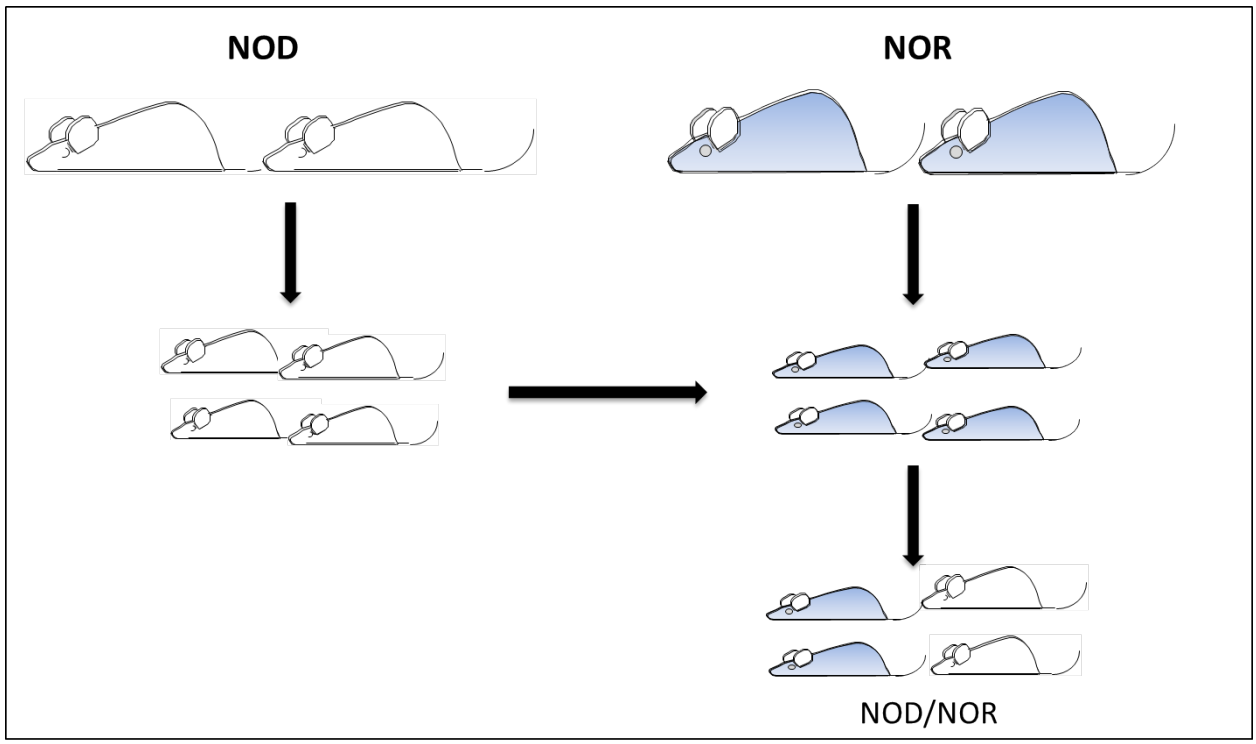


Figure 9. Breeding pairs of NOD and NOR mice were simultaneously set up. At birth half of each litter was switched with the opposite strain and was then nursed by a mother of a different strain. Littermates therefore consisted of mice born to that mother along with mice from another birthing mother. At weaning pups were separated based on sex and nursing mother.

A)

B)

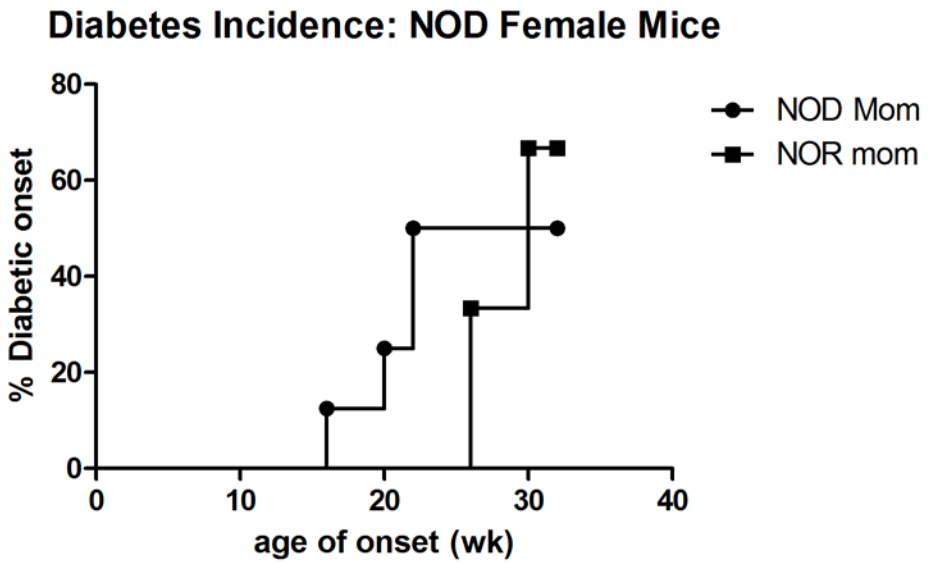
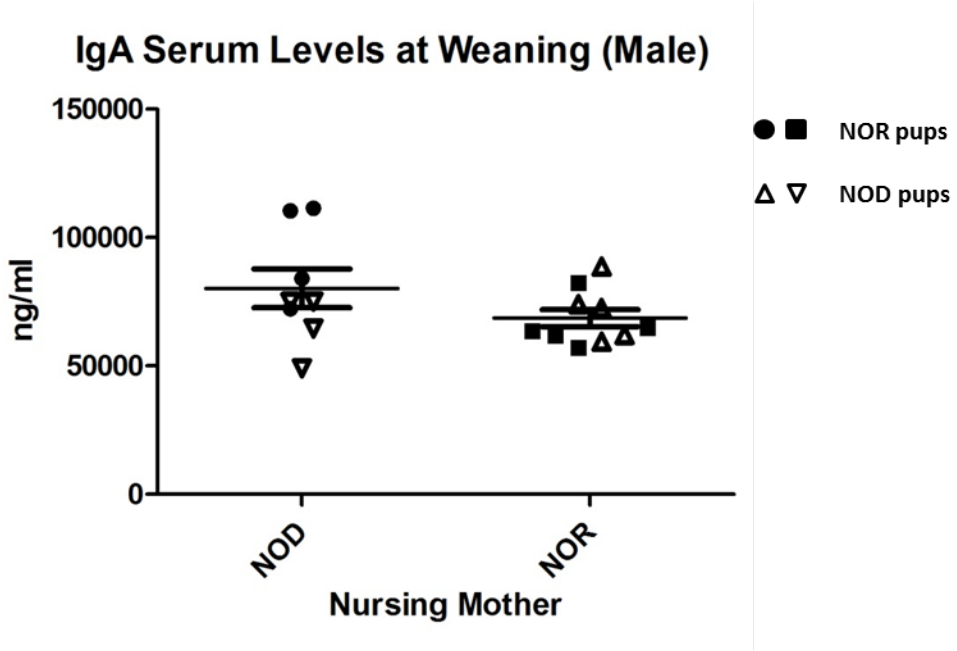


Figure 10. Diabetes incidence male NOD mice raised by a NOD mother (n=4) and NOR mother (n=10) (A). Diabetes incidence female NOD mice raised by a NOD mother (n=8) and NOR mother (n=3) (B).

A)



B)

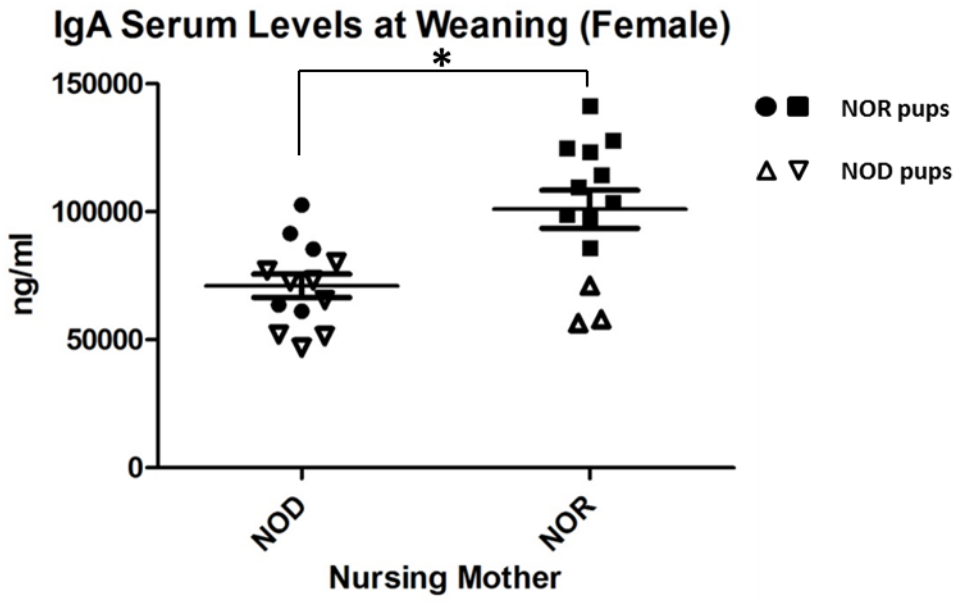


Figure 11. Total IgA levels in the serum between mice, combined male and female, nursed by a NOD (n=21) and NOR (n=23)mother are not significant (p=0.08) (A). Total IgA levels between female mice that have been nursed by a NOD (n=13) and NOR (n=13) mother are significant ($p<0.005$) (B). Total IgA levels in the serum between male mice nursed by a NOD (n=8) and NOR (n=10) mother are not significant (C).

UNPUBLISHED DATA

Introduction

The GI ecosystem is a stable alliance between resident microbiota, immune mediators, and the epithelial barrier (1). If the normal composition of the resident microbiota is altered, it can affect the humoral immune response, as well as the functional state of peripheral T lymphocytes (2-4). Intriguingly, it has now been shown that the pancreatic lymph nodes (PLNs) can sample not only self-antigens from the pancreas, but are also exposed to microbial products from the GI track (5). In a normal mouse (or human), there appears to be tight mucosal compartmentalization of microbial antigens by the mucosal immune system, which limits this systemic exposure (6). However, during intestinal inflammation (colitis) or in animals/humans who have altered intestinal barrier permeability (T1D), the microbial products can freely cross the intestinal barrier and reach draining lymph nodes, including PLNs (7). This has clearly been demonstrated in patients with T1D, where systemic immune responses to cow's milk proteins have been observed (8). Our laboratory has now shown that some NOD mice have detectable serum levels of antibodies to microbial products, including flagellin. However, it is unknown whether these autoantibodies are just biomarkers of altered intestinal permeability or if these autoantibodies are actually mechanistically altering the immune response to autoantigens and therefore their presence can help predict the subsequent development (or lack of development) of T1D.

Results

We have identified a unique antigen from cecal content that was recognized by antibodies from the serum of a diabetic female NOD mouse that was not seen in an age matched NOR mouse (Fig. 1). Mass spectrometry revealed that this band contained multiple bacterial antigens, some of which included flagellin (Table 1).

CBir1 flagellin is from a commensal bacterium and has shown to have a correlation with active IBD (9, 10). Due to its association with an inflammatory autoimmune disease, we decided to investigate its role in T1D. To test the potential protective effect of CBir1 flagellin antibodies, IgG and IgM antibodies against CBir1 flagellin (generously donated by C.O. Elson) were injected into NOD and NOR pups starting at 10 days of age. Previous research from Mathis et al., shows that pancreatic remodeling, programmed cell death, occurs around 10 days of age and it is at this point that antigen presenting cells (APCs) activate diabetogenic T-cells (11).

We hypothesized that the presence of anti-CBir1 flagellin antibodies at this time of pancreatic remodeling would interfere with APC activation of diabetogenic T-cells. This interruption in T-cell activation would then lead to β-cell survival and a reduction in T1D incidence. Probing of pancreatic antigens with the CBir1 flagellin antibodies 3b3 (IgG) and 2B5 (IgM) showed that 2B5 not only recognizes flagellin (Fla2), but also recognized pancreatic antigens (Fig. 2). However, 3b3 fails to recognize pancreatic antigens and only recognizes flagellin (Fla2). Multiple studies in which female and male NOD/NOR mice were injected with either anti-CBir1 flagellin IgG or IgM, revealed no reduction in T1D incidence (Fig. 3).

Discussion

The failure of anti-CBir1 flagellin antibodies to alter disease incidence does not preclude the potential of antibodies to other luminal antigens to induce or protect from diabetes pathogenesis. Antibodies to CBir1 flagellin can still serve as an indicator of intestinal permeability. Further investigation is required to determine if antibodies to commensal bacteria can also serve biomarkers are for susceptibility to progress to develop T1D.

References

1. McCracken V, Lorenz R. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. Cellular microbiology. 2001;3(1):1-11. PubMed PMID: 11207615.
2. Huang T, Wei B, Velazquez P, Borneman J, Braun J. Commensal microbiota alter the abundance and TCR responsiveness of splenic naïve CD4+ T lymphocytes. Clin Immunol. 2005;117(3):221-30. PubMed PMID: 16290233.
3. Dimmitt RA, Staley EM, Chuang G, Tanner SM, Soltau TD, Lorenz RG. Role of postnatal acquisition of the intestinal microbiome in the early development of immune function. Journal of pediatric gastroenterology and nutrition. 2010;51(3):262-73. doi: 10.1097/MPG.0b013e3181e1a114. PubMed PMID: 20639773; PubMed Central PMCID: PMC2932839.
4. Brisbin JT, Gong J, Orouji S, Esufali J, Mallick AI, Parvizi P, Shewen PE, Sharif S. Oral treatment of chickens with lactobacilli influences elicitation of immune responses. Clin Vaccine Immunol. 2011. Epub 2011/07/08. doi: 10.1128/CVI.05100-11. PubMed PMID: 21734067.
5. Turley SJ, Lee JW, Dutton-Swain N, Mathis D, Benoist C. Endocrine self and gut non-self intersect in the pancreatic lymph nodes. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(49):17729-33. Epub 2005/12/01. doi: 10.1073/pnas.0509006102. PubMed PMID: 16317068; PubMed Central PMCID: PMC1308925.
6. Konrad A, Cong Y, Duck W, Borlaza R, Elson C. Tight mucosal compartmentation of the murine immune response to antigens of the enteric microbiota. Gastroenterology. 2006;130(7):2050-9. PubMed PMID: 16762628.
7. Natividad JM, Huang X, Slack E, Jury J, Sanz Y, David C, Denou E, Yang P, Murray J, McCoy KD, Verdu EF. Host responses to intestinal microbial antigens in gluten-sensitive mice. PloS one. 2009;4(7):e6472. Epub 2009/08/04. doi: 10.1371/journal.pone.0006472. PubMed PMID: 19649259; PubMed Central PMCID: PMC2715133.
8. Luopajarvi K, Savilahti E, Virtanen SM, Ilonen J, Knip M, Akerblom HK, Vaarala O. Enhanced levels of cow's milk antibodies in infancy in children who develop type 1 diabetes later in childhood. Pediatr Diabetes. 2008;9(5):434-41. Epub 2008/05/28. doi: 10.1111/j.1399-5448.2008.00413.x. PubMed PMID: 18503496.
9. Lodes MJ, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, Fort M, Hershberg RM. Bacterial flagellin is a dominant antigen in Crohn disease. The Journal of clinical investigation. 2004;113(9):1296-306. doi: 10.1172/JCI20295. PubMed PMID: 15124021; PubMed Central PMCID: PMC398429.
10. Duck LW, Walter MR, Novak J, Kelly D, Tomasi M, Cong Y, Elson CO. Isolation of flagellated bacteria implicated in Crohn's disease. Inflammatory bowel diseases. 2007;13(10):1191-201. doi: 10.1002/ibd.20237. PubMed PMID: 17712838.
11. Turley S, Poirot L, Hattori M, Benoist C, Mathis D. Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model. The Journal of experimental medicine. 2003;198(10):1527-37. doi: 10.1084/jem.20030966. PubMed PMID: 14623908; PubMed Central PMCID: PMC2194112.

Table 1. Identification of luminal bacterial antigen by serum of female diabetic NOD mouse.

Bacterial Antigen	Mowse Score
pyruvate, phosphate dikinase [Eubacterium rectale =ATCC=20 33656]	392
FlaA [Clostridium =botulinum]	306
hypothetical protein CLOHYLEM_06095 [Clostridium =hylemonae=20 DSM 15053]	297
putative RagA protein [Prevotella copri DSM=20 18205]	199
flagellin [Butyrivibrio crossotus DSM=20 2876]	198
Fla3 flagellin [Lachnospiraceae bacterium=20 A4]	161
hypothetical protein B2_05005 [Bacteroides sp.=20 2_1_7]	131
hypothetical protein BACUNI_00914 [Bacteroides =uniformis=20 ATCC 8492]	123
pyruvate:ferredoxin (flavodoxin) oxidoreductase =[Prevotella=20 sp. oral taxon 317 str. F0108]	120
putative outer membrane protein [Pedobacter sp.=20 BAL39]	114
hypothetical protein BACUNI_00842 [Bacteroides =uniformis=20 ATCC 8492]	111
thioredoxin [Escherichia =coli]	105
hypothetical protein BVU_1841 [Bacteroides vulgatus =ATCC=20 8482]	98
hypothetical protein PRU_2734 [Prevotella ruminicola=20 23]	90

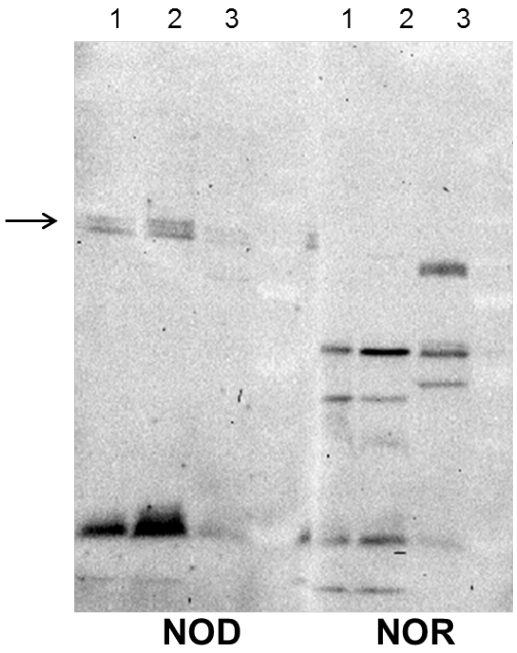
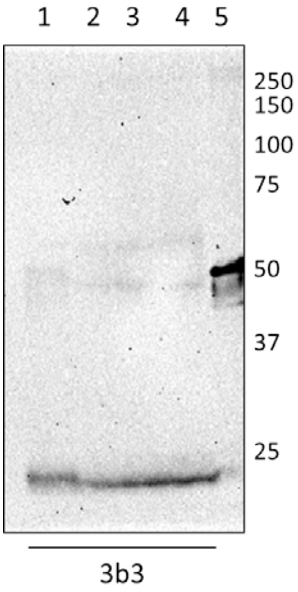


Figure 1. Cecal probe using serum from a female diabetic NOD mouse (25wks) and age matched NOR mouse. Lanes 1-3, contained cecal protein from 30 week male NOD mice (1-2) and 12 week old male NOD mice (3). A band was identified at approximately 100 kD in samples probed with NOD serum that was not seen in samples probed with NOR serum.

A)



B)

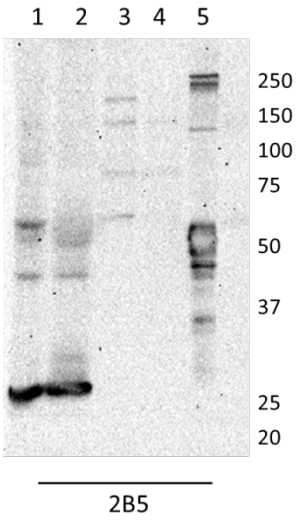
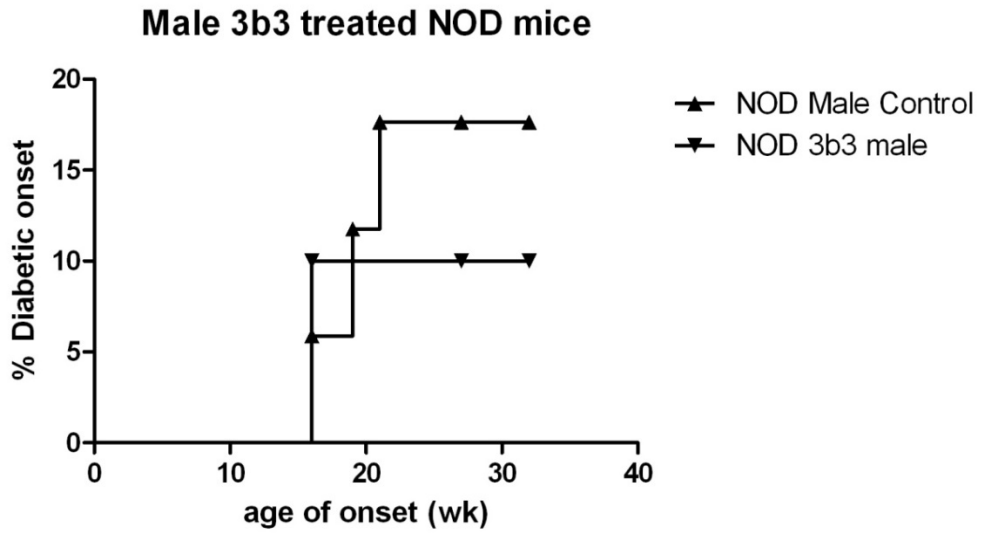
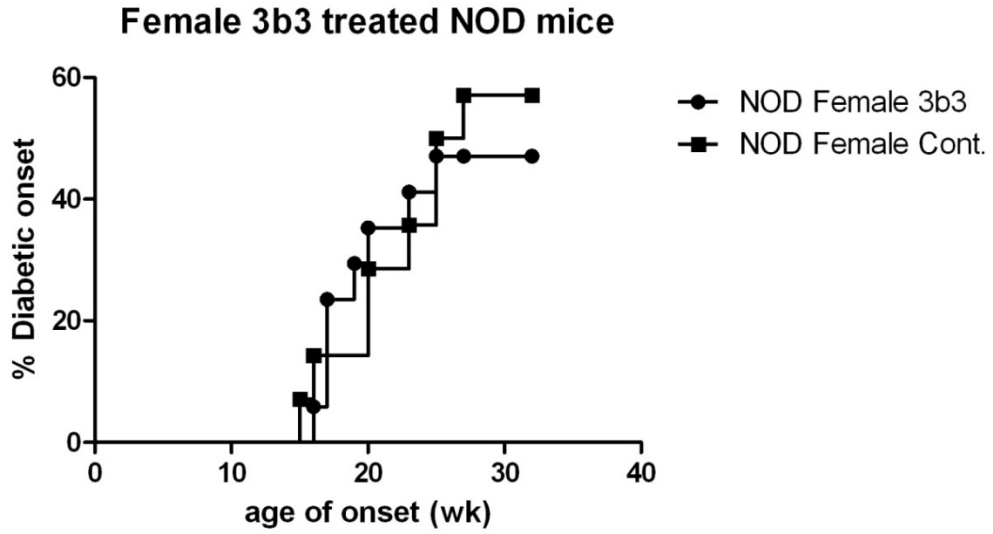


Figure 2. Pancreatic antigens and flagellin were probed with the CBir1 flagellin antibodies 3b3 (IgG) and 2B5 (IgM). (A) 3b3 did not recognize pancreatic antigens (1. 2wk NOD pancreas, 2. 9wk NOD pancreas, 3. 9wk NOR pancreas, 4. 10wk H_2g7), but did recognize flagellin (5. Fla 2). (B) 2B5 did recognize pancreatic antigens (1. 10wk NOD, 2. 9wk H_2G7, 3. 4wk NOR islet lysate, 4. 5wk H_2g7 islet lysate) and also recognized flagellin (5. Fla 2).

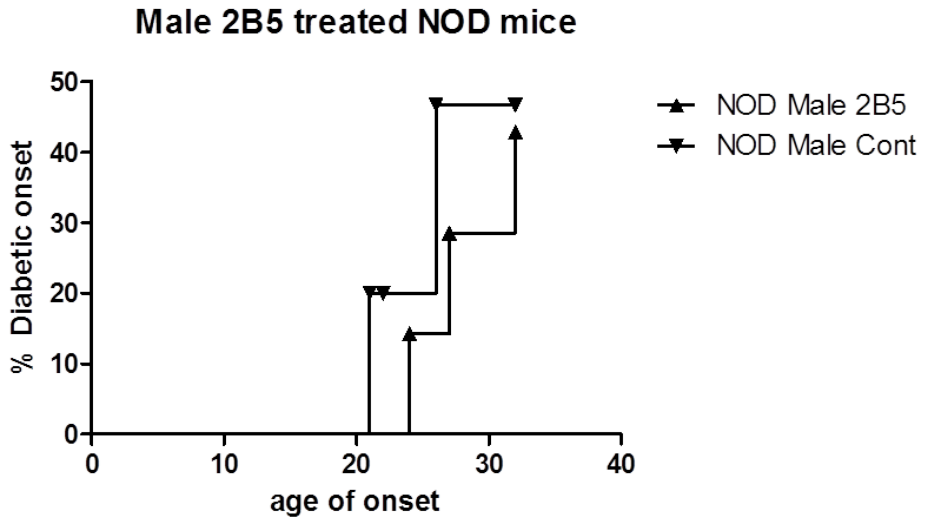
A)


Male 3b3 treated NOD mice
% Diabetic onset
age of onset (wk)
NOD Male Control
NOD 3b3 male

B)


Female 3b3 treated NOD mice
% Diabetic onset
age of onset (wk)
NOD Female 3b3
NOD Female Cont.

C)



D)

Figure 3. There was no significant reduction in diabetes in NOD male or female mice treated with the CBir1 flagellin antibodies 3b3 (IgG) or 2B5 (IgM). (A) When compared to controls (n=21) male NOD mice injected with 3b3 (n=11) did not have a significant reduction in T1D incidence. (B) When compared to controls (n=18) female NOD mice injected with 3b3 (n=19) did not have a significant reduction in T1D incidence. (C) When compared to controls (n=9) male NOD mice injected with 2B5 (n=9) did not have a significant reduction in T1D. (D) When compared to controls (n=9) NOD female mice (n=11) injected with 2B5 did not have a significant reduction in T1D.

CONCLUSIONS

Our appreciation of the role the GI ecosystem plays in shaping the immune system has grown as data continues to show that there are variations in healthy individuals and individuals that are predisposed to disease. In animal models and human subjects with T1D there are alterations to intestinal barrier integrity and alterations to the intestinal microbiota. Current controls for T1D often include C57BL/6 and BALB/c mice, which do not share these abnormalities in their GI system that we see in the commonly used NOD model. To better understand disease progression a control model is needed that mimics some of these GI abnormalities seen in the NOD mouse.

To address this issue, we have proposed the use of the diabetes resistant NOR mouse as a control model. The NOR mouse shares the common polymorphism of the MHCII ($H2g^7$) gene that is seen in animal models and humans along with other diabetic loci. Although, the NOR mouse develops peri-insulitis like diabetic mice and humans, the peri-insulitis fails to progress to severe insulitis and NOR mice do not develop diabetes. Importantly our data shows that NOR mice, like NOD mice, have a permeable intestinal barrier that we do not see in other commonly used controls. However, this is contradictory to one report that sees a decrease in intestinal permeability in 12 week NOR mice compared to age matched NOD mice [22]. Comparison between the NOD and NOR strains will reveal mechanisms of disease pathogenesis and protection.

To better understand what and how luminal antigens are affecting disease incidence we performed a series of Western's where we used serum from NOD and NOR mice as probes to determine the presence of an immune reaction to bacterial antigens

from the lumen of the cecum. These probes allowed us to analyze and determine, via mass spectrometry , the identity of luminal antigens that are crossing the intestinal barrier and inducing immune responses in NOD and NOR mice. When comparing immune reactivity between a diabetic NOD mouse and an age matched NOR mouse we identified a band that was unique to the NOD mouse. Within this unique band, multiple commensal bacterial flagellin were identified. One of these bacterial flagellin was the CBir1 flagellin, which is a known antigen that is associated with disease pathogenesis in inflammatory bowel disease [143]. After the identification of CBir1 flagellin as a potential diabetogenic antigen, serum analysis of prediabetic NOD mice revealed a significantly reduced level of CBir1 flagellin antibodies when compared to diabetic resistant NOR mice, as measured by anti-CBir1 flagellin IgG. This shows that the presence of anti-CBir1 flagellin may in fact be protective and not pathogenic as previously thought when analyzing the serum of a diabetic NOD mouse.

As previously mentioned, multiple groups have shown a difference in commensal microbiota between healthy and individuals predisposed to develop disease [137, 144-148]. Multiple groups have focused how these differences in commensal microbiota are altering the immune system. Little research has focused on what causes this baseline difference in microbiota. We have shown that in the presence of bacteria in the GI tract there is a difference in expression levels (mRNA) of AMPs and mucins between NOD and NOR mice at 2 and 5 weeks. This is not surprising when coupled with the differences in commensal microbiota between the two strains of mice. A difference in microbiota may lead to a difference in expression of AMPs and mucins between NOD and NOR mice. However, it is also possible that expression levels are constitutive and

are not dictated by bacteria presence or absence. We have now shown that when NOD and NOR mice are treated with antibiotics to remove bacteria from their GI tracts (simulating germfree conditions) a difference is still seen in the expression of AMPs and mucins. At 2 weeks of age, NOD mice that have been raised on antibiotics have a significantly reduced expression of MMP7 and MUC2 when compared to NOR mice. The reduction in MMP7 expression is of great importance because it is needed to activate the major class of AMPs known as defensins. Without the activation of AMPs, such as defensins, there will be an alteration to commensal microbiota. MUC2 is also the major component of intestinal mucus and alterations of expression could have a significant effect on what is colonizing the gut. This indicates that NOR mice may have a higher expression level of key components to barrier defense and may be more capable of controlling the bacterial species that are present in their GI tract, specifically their distal ileum. We feel that a reduction in expression of MMP7 and MUC2 in NOD mice leads to a higher susceptibility of colonization by a commensal microbiota that predisposes them to disease.

Data showing a reduction in expression of MMP7 and MUC2 in the absence of GI bacteria was further supported by the functional "mucus kill assay". At 5 weeks when the mucus layer from the distal ileum was removed and cultured with *E. coli* and then plated, the cfu's were significantly reduced in NOR mice compared to NOD mice. However there was no significant reduction in cfu's at 10 weeks between the two mouse strains. This data reveals two important facts: 1) difference in ability to regulate bacteria occurs early in life and dissipates as mice age and 2) the "mucus kill assay" supports variations seen in the mRNA expression of AMPs, mucins, and related peptides.

The appreciation of the role of microbiota makes it an important target in treating autoimmune diseases, such as T1D. Currently alterations to microbiota, through fecal transplants, are being successfully utilized to treat *C. difficle* infections in patients. We think it is of great importance to consider that *C. difficle* patients that have undergone successful fecal transplant had previously had a healthy microbiota before disease onset. Data is still be collected on the long term effects and mechanisms by which fecal transplants are helping patients recover. It is likely that fecal transplants are simply allowing a reset to a healthy commensal microbiota, replacing the disease causing *C. difficle*. Our data shows that baseline expression of AMPs and mucins in the absence of bacteria in NOD mice are reduced compared to NOR mice. Therefore baseline expression of AMPs and mucins may not be able to support a healthy microbiota once it has been introduced and the host may revert back to their microbiota that predisposes them to disease.

We propose that future research focus on manipulating the expression of AMPs and mucins as a means of promoting a healthy microbiota in a diseased host. Currently fecal transplants or the use of antibiotics are the only methods used in human subjects to manipulate the microbiota. As previously mentioned, potential agents that could be used to modify AMP and mucin expression are bethanechol and vanadate. Vanadate has been shown to increase MUC1 in HES cells and MUC5AC in respiratory epithelial cells, while bethanechol stimulates Paneth cells and has been shown to increase MMP7 expression at the message level [72, 86-90]. Importantly, besides their potential ability to modify the commensal microbiota through the expression of AMPs and mucins, both drugs have been shown to lower blood glucose levels in NOD mice, rats and humans [82, 84, 85,

91]. Future studies must be done to see if using these drugs could modify the commensal microbiota and prevent disease pathogenesis through stimulation of AMPs and mucins. Treatments such as this would allow for an alternative to treatments such as fecal transplants.

The difference in the microbiota between NOD and NOR may be exaggerated by variations in cell turnover in the intestinal crypts where many AMPs are produced. BrdUrd staining of crypts in the ileums of NOD and NOR mice at 2 and 10 weeks reveals a significantly higher number of dividing cells per crypt in NOR mice. This could explain the higher level of expression of AMPs and mucins seen in NOR mice. The ability to respond to intestinal injury induced by pathogenic microbiota or other factors may affect disease susceptibility. To induce intestinal injury lethal radiation was used. NOR mice that are lethally irradiated have a significantly higher number of regenerative crypts, as measured by BrdUrd staining, when compared to NOD mice. NOD mice are hindered in responding to intestinal insults and therefore may be more susceptible to antigens crossing the epithelial barrier causing sustained immune responses and disease pathogenesis. The ability of NOR mice to respond to injury could explain how they are protected from disease in the harsh GI ecosystem.

To transfer microbiota between individual animals, multiple techniques have been employed that often require a large number of animals and repeated delivery of bacteria to induce a microbial shift. Common methods used for animal models include, antibiotics, gavage of fecal content, co-housing [121, 131, 140-142]. Another potential way to introduce a healthy commensal microbiota into animal models that are predisposed to disease, is through cross-fostering. Cross-fostering is done in an attempt

to override the commensal microbiota that is introduced during vaginal birth and replace it with commensal microbiota from the nursing mother. Therefore NOD mice that are susceptible to develop diabetes can have their microbiota replaced by being nursed by diabetic resistant NOR mice. Our data in mice indicates that it is possible to induce a permanent shift in the commensal microbiota of diabetes susceptible NOD pups if they are nursed by a diabetic resistant NOR mother. Mice group tightly based on bacterial phylums found in their fecal pellets at weaning (4wks) and although there is a shift in bacterial phylums at 32 weeks, groupings remained the same. Our sample size is not large enough to accurately determine the beneficial effects of a NOR microbiota in female NOD mice. However, in male NOD mice that have been nursed by a NOR mother we see a reduction in the incidence of diabetes when compared to male mice that have been nursed by a NOD mother. Further studies are required to determine if and how NOR microbiota is protecting NOD mice from developing diabetes.

Although NOR microbiota has been successfully transferred into NOD for the span of their lives, it is unknown if NOD mice have taken on other NOR phenotypes. As previously mentioned we reported an increase in BrdUrd positive cells in the crypts of the distal ileum of NOR mice compared to NOD mice. We have also shown an increased response to intestinal injury after lethal irradiation as measured by regenerative crypts. If NOD mice have been colonized by NOR commensal microbiota they may take on characteristics that are typically seen in NOR mice. Further studies will be required to analyses these questions, determining the effects of NOR microbiota in NOD mice.

Type 1 diabetes is a polygenic disease with unknown environmental triggers, one of these environmental triggers may be commensal microbiota. There is a clear

distinction between the microbiota of healthy and individuals predisposed to disease. The NOR mouse represents an essential control for T1D animal studies, due to similar genetic backgrounds along with its permeable intestinal barrier. Manipulation of the microbiota may serve as a viable treatment for T1D and other autoimmune diseases. Although manipulation through cross-fostering is not highly applicable to human subjects, it does allow for the transfer of NOR (diabetic) resistant microbiota to NOD mice. This transfer last for the lifetime of the mice, but it's effectiveness in treating T1D needs further exploration. Further studies comparing NOD and NOR mice are needed to better understand disease pathogenesis and protective mechanisms.

As our knowledge of how to effectively transfer microbiota in animal models along with our understanding of long term microbial shifts continues to progress. The ultimate goal of these studies is to translate these methods of altering the microbiota in animal models into treatments for humans that are predisposed to disease such as T1D. Although cross-fostering human infants is likely not going to be an accepted treatment for preventing disease, its use in animal models does highlight the importance of the mothers microbiota in influencing colonization in offspring. Treatments in the future may involve altering the microbiota, either through antibiotics or fecal transplant, of expecting mothers as a way of ensuring colonization by a healthy microbiota in newborns. Colonization by a healthy microbiota could then lower disease incidence later in life.

GENERAL LIST OF REFERENCES

[1] Kamada N, Nunez G (2014) Regulation of the Immune System by the Resident Intestinal Bacteria. Gastroenterology

[2] Lee YK, Mazmanian SK (2010) Has the microbiota played a critical role in the evolution of the adaptive immune system? Science 330: 1768-1773

[3] Tiwana H, Wilson C, Walmsley RS, et al. (1997) Antibody responses to gut bacteria in ankylosing spondylitis, rheumatoid arthritis, Crohn's disease and ulcerative colitis. Rheumatology international 17: 11-16

[4] Wu HJ, Ivanov, II, Darce J, et al. (2010) Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity 32: 815-827

[5] Conte MP, Schippa S, Zamboni I, et al. (2006) Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease. Gut 55: 1760-1767

[6] Ray K (2012) Microbiota: Tolerating gluten--a role for gut microbiota in celiac disease? Nature reviews Gastroenterology & hepatology 9: 242

[7] Vaarala O, Atkinson MA, Neu J (2008) The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. Diabetes 57: 2555-2562

[8] Atkinson MA, Eisenbarth GS, Michels AW (2014) Type 1 diabetes. Lancet 383: 69-82

[9] Dahlquist G, Savilahti E, Landin-Olsson M (1992) An increased level of antibodies to beta-lactoglobulin is a risk determinant for early-onset type 1 (insulin-dependent) diabetes mellitus independent of islet cell antibodies and early introduction of cow's milk. Diabetologia 35: 980-984

[10] Cardwell CR, Stene LC, Ludvigsson J, et al. (2012) Breast-feeding and childhood-onset type 1 diabetes: a pooled analysis of individual participant data from 43 observational studies. Diabetes care 35: 2215-2225

[11] Schuppan D, Hahn EG (2001) Celiac disease and its link to type 1 diabetes mellitus. Journal of pediatric endocrinology & metabolism : JPEM 14 Suppl 1: 597-605

[12] Turley SJ, Lee JW, Dutton-Swain N, Mathis D, Benoist C (2005) Endocrine self and gut non-self intersect in the pancreatic lymph nodes. Proceedings of the National Academy of Sciences of the United States of America 102: 17729-17733

[13] Carter PB, Collins FM (1974) The route of enteric infection in normal mice. The Journal of experimental medicine 139: 1189-1203

[14] Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA (2008) Mucins in the mucosal barrier to infection. Mucosal immunology 1: 183-197

[15] Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y (1980) Breeding of a non-obese, diabetic strain of mice. Jikken dobutsu Experimental animals 29: 1-13

[16] Serreze DV, Chapman HD, Varnum DS, Gerling I, Leiter EH, Shultz LD (1997) Initiation of autoimmune diabetes in NOD/Lt mice is MHC class I-dependent. Journal of immunology 158: 3978-3986

[17] Marliss EB, Nakhooda AF, Poussier P, Sima AA (1982) The diabetic syndrome of the 'BB' Wistar rat: possible relevance to type 1 (insulin-dependent) diabetes in man. Diabetologia 22: 225-232

[18] Wallis RH, Wang K, Marandi L, et al. (2009) Type 1 diabetes in the BB rat: a polygenic disease. Diabetes 58: 1007-1017

[19] Noble JA, Erlich HA (2012) Genetics of type 1 diabetes. Cold Spring Harbor perspectives in medicine 2: a007732

[20] Vaarala O (2008) Leaking gut in type 1 diabetes. Current opinion in gastroenterology 24: 701-706

[21] Neu J, Reverte CM, Mackey AD, et al. (2005) Changes in intestinal morphology and permeability in the biobreeding rat before the onset of type 1 diabetes. Journal of pediatric gastroenterology and nutrition 40: 589-595

[22] Lee AS, Gibson DL, Zhang Y, Sham HP, Vallance BA, Dutz JP (2010) Gut barrier disruption by an enteric bacterial pathogen accelerates insulitis in NOD mice. Diabetologia 53: 741-748

[23] Heyman M, Abed J, Lebreton C, Cerf-Bensussan N (2012) Intestinal permeability in coeliac disease: insight into mechanisms and relevance to pathogenesis. Gut 61: 1355-1364

[24] Sapone A, de Magistris L, Pietzak M, et al. (2006) Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. Diabetes 55: 1443-1449

[25] Bosi E, Molteni L, Radaelli MG, et al. (2006) Increased intestinal permeability precedes clinical onset of type 1 diabetes. Diabetologia 49: 2824-2827

[26] Menard S, Cerf-Bensussan N, Heyman M (2010) Multiple facets of intestinal permeability and epithelial handling of dietary antigens. Mucosal immunology 3: 247-259

[27] Schumann M, Richter JF, Wedell I, et al. (2008) Mechanisms of epithelial translocation of the alpha(2)-gliadin-33mer in coeliac sprue. Gut 57: 747-754

[28] Mack DR, Ahrne S, Hyde L, Wei S, Hollingsworth MA (2003) Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. Gut 52: 827-833

[29] McGuckin MA, Linden SK, Sutton P, Florin TH (2011) Mucin dynamics and enteric pathogens. Nature reviews Microbiology 9: 265-278

[30] Garrett WS, Gordon JI, Glimcher LH (2010) Homeostasis and inflammation in the intestine. Cell 140: 859-870

[31] Buffie CG, Pamer EG (2013) Microbiota-mediated colonization resistance against intestinal pathogens. Nature reviews Immunology 13: 790-801

[32] Seekatz AM, Aas J, Gessert CE, et al. (2014) Recovery of the Gut Microbiome following Fecal Microbiota Transplantation. mBio 5

[33] Salzman NH, Hung K, Haribhai D, et al. (2010) Enteric defensins are essential regulators of intestinal microbial ecology. Nature immunology 11: 76-83

[34] Mann ER, Landy JD, Bernardo D, et al. (2013) Intestinal dendritic cells: their role in intestinal inflammation, manipulation by the gut microbiota and differences between mice and men. Immunology letters 150: 30-40

[35] Nutsch KM, Hsieh CS (2012) T cell tolerance and immunity to commensal bacteria. Current opinion in immunology 24: 385-391

[36] Leon F, Smythies LE, Smith PD, Kelsall BL (2006) Involvement of dendritic cells in the pathogenesis of inflammatory bowel disease. Advances in experimental medicine and biology 579: 117-132

[37] Mortha A, Chudnovskiy A, Hashimoto D, et al. (2014) Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. Science 343: 1249288

[38] Sedda S, Marafini I, Figliuzzi MM, Pallone F, Monteleone G (2014) An overview of the role of innate lymphoid cells in gut infections and inflammation. Mediators of inflammation 2014: 235460

[39] Teshima CW, Dieleman LA, Meddings JB (2012) Abnormal intestinal permeability in Crohn's disease pathogenesis. Annals of the New York Academy of Sciences 1258: 159-165

[40] Mooradian AD, Morley JE, Levine AS, Prigge WF, Gebhard RL (1986) Abnormal intestinal permeability to sugars in diabetes mellitus. Diabetologia 29: 221-224

[41] Arrieta MC, Bistritz L, Meddings JB (2006) Alterations in intestinal permeability. Gut 55: 1512-1520

[42] Groschwitz KR, Hogan SP (2009) Intestinal barrier function: molecular regulation and disease pathogenesis. The Journal of allergy and clinical immunology 124: 3-20; quiz 21-22

[43] Meddings JB, Jarand J, Urbanski SJ, Hardin J, Gall DG (1999) Increased gastrointestinal permeability is an early lesion in the spontaneously diabetic BB rat. The American journal of physiology 276: G951-957

[44] Valladares R, Sankar D, Li N, et al. (2010) Lactobacillus johnsonii N6.2 mitigates the development of type 1 diabetes in BB-DP rats. PloS one 5: e10507

[45] Visser JT, Lammers K, Hoogendijk A, et al. (2010) Restoration of impaired intestinal barrier function by the hydrolysed casein diet contributes to the prevention of type 1 diabetes in the diabetes-prone BioBreeding rat. Diabetologia 53: 2621-2628

[46] Gibson DL, Ma C, Bergstrom KS, Huang JT, Man C, Vallance BA (2008) MyD88 signalling plays a critical role in host defence by controlling pathogen burden and promoting epithelial cell homeostasis during Citrobacter rodentium-induced colitis. Cellular microbiology 10: 618-631

[47] Wang W, Uzzau S, Goldblum SE, Fasano A (2000) Human zonulin, a potential modulator of intestinal tight junctions. J Cell Sci 113 Pt 24: 4435-4440

[48] Watts T, Berti I, Sapone A, et al. (2005) Role of the intestinal tight junction modulator zonulin in the pathogenesis of type I diabetes in BB diabetic-prone rats. Proceedings of the National Academy of Sciences of the United States of America 102: 2916-2921

[49] Tripathi A, Lammers KM, Goldblum S, et al. (2009) Identification of human zonulin, a physiological modulator of tight junctions, as prehaptoglobin-2. Proceedings of the National Academy of Sciences of the United States of America 106: 16799-16804

[50] Neu J, Reverte CM, Mackey AD, et al. (2005) Changes in intestinal morphology and permeability in the biobreeding rat before the onset of type 1 diabetes. Journal of pediatric gastroenterology and nutrition 40: 589-595

[51] Mariadason JM, Barkla DH, Gibson PR (1997) Effect of short-chain fatty acids on paracellular permeability in Caco-2 intestinal epithelium model. The American journal of physiology 272: G705-712

[52] Li N, Hatch M, Wasserfall CH, et al. (2010) Butyrate and type 1 diabetes mellitus: can we fix the intestinal leak? J Pediatr Gastroenterol Nutr 51: 414-417

[53] Gao Z, Yin J, Zhang J, et al. (2009) Butyrate improves insulin sensitivity and increases energy expenditure in mice. Diabetes 58: 1509-1517

[54] Fasano A, Not T, Wang W, et al. (2000) Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. Lancet 355: 1518-1519

[55] Courtois P, Nsimba G, Jijakli H, Sener A, Scott FW, Malaisse WJ (2005) Gut permeability and intestinal mucins, invertase, and peroxidase in control and diabetes-prone BB rats fed either a protective or a diabetogenic diet. Digestive diseases and sciences 50: 266-275

[56] Patrick C, Wang GS, Lefebvre DE, et al. (2013) Promotion of autoimmune diabetes by cereal diet in the presence or absence of microbes associated with gut immune activation, regulatory imbalance, and altered cathelicidin antimicrobial Peptide. Diabetes 62: 2036-2047

[57] Hansen AK, Ling F, Kaas A, Funda DP, Farlov H, Buschard K (2006) Diabetes preventive gluten-free diet decreases the number of caecal bacteria in non-obese diabetic mice. Diabetes/metabolism research and reviews 22: 220-225

[58] Alam C, Valkonen S, Palagani V, Jalava J, Eerola E, Hanninen A (2010) Inflammatory tendencies and overproduction of IL-17 in the colon of young NOD mice are counteracted with diet change. Diabetes 59: 2237-2246

[59] Turley S, Poirot L, Hattori M, Benoist C, Mathis D (2003) Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model. The Journal of experimental medicine 198: 1527-1537

[60] Carter PB, Collins FM (1974) The route of enteric infection in normal mice. The Journal of experimental medicine 139: 1189-1203

[61] Turley SJ, Lee JW, Dutton-Swain N, Mathis D, Benoist C (2005) Endocrine self and gut non-self intersect in the pancreatic lymph nodes. Proceedings of the National Academy of Sciences of the United States of America 102: 17729-17733

[62] Ganz T (2002) Immunology. Versatile defensins. Science 298: 977-979

[63] Eisenhauer PB, Harwig SS, Szklarek D, Ganz T, Selsted ME, Lehrer RI (1989) Purification and antimicrobial properties of three defensins from rat neutrophils. Infection and immunity 57: 2021-2027

[64] Holmen Larsson JM, Thomsson KA, Rodriguez-Pineiro AM, Karlsson H, Hansson GC (2013) Studies of mucus in mouse stomach, small intestine, and colon. III. Gastrointestinal Muc5ac and Muc2 mucin O-glycan patterns reveal a regiospecific distribution. American journal of physiology Gastrointestinal and liver physiology 305: G357-363

[65] Kim YS, Ho SB (2010) Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep 12: 319-330

[66] Fueger PT, Schisler JC, Lu D, et al. (2008) Trefoil factor 3 stimulates human and rodent pancreatic islet beta-cell replication with retention of function. Molecular endocrinology 22: 1251-1259

[67] Orime K, Shirakawa J, Togashi Y, et al. (2013) Trefoil factor 2 promotes cell proliferation in pancreatic beta-cells through CXCR-4-mediated ERK1/2 phosphorylation. Endocrinology 154: 54-64

[68] Eckmann L (2005) Defence molecules in intestinal innate immunity against bacterial infections. Current opinion in gastroenterology 21: 147-151

[69] Cunliffe RN (2003) Alpha-defensins in the gastrointestinal tract. Molecular immunology 40: 463-467

[70] Kaiser V, Diamond G (2000) Expression of mammalian defensin genes. Journal of leukocyte biology 68: 779-784

[71] Cunliffe RN, Mahida YR (2004) Expression and regulation of antimicrobial peptides in the gastrointestinal tract. J Leukoc Biol 75: 49-58

[72] Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ (2000) Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nature immunology 1: 113-118

[73] Underwood MA, Kananurak A, Coursodon CF, et al. (2012) Bifidobacterium bifidum in a rat model of necrotizing enterocolitis: antimicrobial peptide and protein responses. Pediatric research 71: 546-551

[74] Ramasundara M, Leach ST, Lemberg DA, Day AS (2009) Defensins and inflammation: the role of defensins in inflammatory bowel disease. Journal of gastroenterology and hepatology 24: 202-208

[75] Nemeth BC, Varkonyi T, Somogyvari F, et al. (2014) Relevance of alpha-defensins (HNP1-3) and defensin beta-1 in diabetes. World journal of gastroenterology : WJG 20: 9128-9137

[76] Iimura M, Gallo RL, Hase K, Miyamoto Y, Eckmann L, Kagnoff MF (2005) Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. Journal of immunology 174: 4901-4907

[77] Chen M, Sun Q, Giovannucci E, et al. (2014) Dairy consumption and risk of type 2 diabetes: 3 cohorts of US adults and an updated meta-analysis. BMC medicine 12: 215

[78] Choudry HA, Mavanur A, O'Malley ME, Zeh HJ, Guo Z, Bartlett DL (2012) Chronic anti-inflammatory drug therapy inhibits gel-forming mucin production in a murine xenograft model of human pseudomyxoma peritonei. Annals of surgical oncology 19: 1402-1409

[79] Ogawa A, Johnson JH, Ohneda M, et al. (1992) Roles of insulin resistance and beta-cell dysfunction in dexamethasone-induced diabetes. The Journal of clinical investigation 90: 497-504

[80] Brown CT, Davis-Richardson AG, Giongo A, et al. (2011) Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes. PLoS One 6: e25792

[81] Courtois P, Jurysta C, Sener A, Scott FW, Malaisse WJ (2005) Quantitative and qualitative alterations of intestinal mucins in BioBreeding rats. Int J Mol Med 15: 105-108

[82] Meyerovitch J, Waner T, Sack J, Kopolovic J, Shemer J (2000) Attempt to prevent the development of diabetes in non-obese diabetic mice by oral vanadate administration. The Israel Medical Association journal : IMAJ 2: 211-214

[83] Mosseri R, Waner T, Shefi M, Shafrir E, Meyerovitch J (2000) Gluconeogenesis in non-obese diabetic (NOD) mice: in vivo effects of vandadate treatment on hepatic glucose-6-phoshatase and phosphoenolpyruvate carboxykinase. Metabolism: clinical and experimental 49: 321-325

[84] Srivastava AK (2000) Anti-diabetic and toxic effects of vanadium compounds. Molecular and cellular biochemistry 206: 177-182

[85] Brichard SM, Desbuquois B, Girard J (1993) Vanadate treatment of diabetic rats reverses the impaired expression of genes involved in hepatic glucose metabolism: effects on glycolytic and gluconeogenic enzymes, and on glucose transporter GLUT2. Molecular and cellular endocrinology 91: 91-97

[86] Yu D, Walters DM, Zhu L, Lee PK, Chen Y (2011) Vanadium pentoxide (V(2)O(5)) induced mucin production by airway epithelium. American journal of physiology Lung cellular and molecular physiology 301: L31-39

[87] Thathiah A, Carson DD (2004) MT1-MMP mediates MUC1 shedding independent of TACE/ADAM17. The Biochemical journal 382: 363-373

[88] Porter EM, Bevins CL, Ghosh D, Ganz T (2002) The multifaceted Paneth cell. Cellular and molecular life sciences : CMLS 59: 156-170

[89] Satoh Y, Ishikawa K, Oomori Y, Takeda S, Ono K (1992) Bethanechol and a G-protein activator, NaF/AlCl3, induce secretory response in Paneth cells of mouse intestine. Cell and tissue research 269: 213-220

[90] Peng Z, Heath J, Drachenberg C, Raufman JP, Xie G (2013) Cholinergic muscarinic receptor activation augments murine intestinal epithelial cell proliferation and tumorigenesis. BMC cancer 13: 204

[91] Fukudo S, Virnelli S, Kuhn CM, Cochrane C, Feinglos MN, Surwit RS (1989) Muscarinic stimulation and antagonism and glucoregulation in nondiabetic and obese hyperglycemic mice. Diabetes 38: 1433-1438

[92] Savilahti E, Ormala T, Saukkonen T, et al. (1999) Jejuna of patients with insulin-dependent diabetes mellitus (IDDM) show signs of immune activation. Clinical and experimental immunology 116: 70-77

[93] Hänninen A, Jaakkola I, Jalkanen S (1998) Mucosal addressin is required for the development of diabetes in nonobese diabetic mice. Journal of immunology 160: 6018-6025

[94] Paronen J, Klemetti P, Kantele J, et al. (1997) Glutamate decarboxylase-reactive peripheral blood lymphocytes from patients with IDDM express gut-specific homing receptor alpha4beta7-integrin. Diabetes 46: 583-588

[95] Jaakkola I, Jalkanen S, Hanninen A (2003) Diabetogenic T cells are primed both in pancreatic and gut-associated lymph nodes in NOD mice. European journal of immunology 33: 3255-3264

[96] Konrad A, Cong Y, Duck W, Borlaza R, Elson C (2006) Tight mucosal compartmentation of the murine immune response to antigens of the enteric microbiota. Gastroenterology 130: 2050-2059

[97] Natividad JM, Huang X, Slack E, et al. (2009) Host responses to intestinal microbial antigens in gluten-sensitive mice. PloS one 4: e6472

[98] Luopajarvi K, Savilahti E, Virtanen SM, et al. (2008) Enhanced levels of cow's milk antibodies in infancy in children who develop type 1 diabetes later in childhood. Pediatr Diabetes 9: 434-441

[99] McCracken V, Lorenz R (2001) The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. Cellular microbiology 3: 1-11

[100] Locke NR, Stankovic S, Funda DP, Harrison LC (2006) TCR gamma delta intraepithelial lymphocytes are required for self-tolerance. Journal of immunology 176: 6553-6559

[101] Todd DJ, Forsberg EM, Greiner DL, Mordes JP, Rossini AA, Bortell R (2004) Deficiencies in gut NK cell number and function precede diabetes onset in BB rats. Journal of immunology 172: 5356-5362

[102] Alam C, Bittoun E, Bhagwat D, et al. (2011) Effects of a germ-free environment on gut immune regulation and diabetes progression in non-obese diabetic (NOD) mice. Diabetologia 54: 1398-1406

[103] Badami E, Sorini C, Coccia M, et al. (2011) Defective differentiation of regulatory FoxP3+ T cells by small-intestinal dendritic cells in patients with type 1 diabetes. Diabetes 60: 2120-2124

[104] Ejsing-Duun M, Josephsen J, Aasted B, Buschard K, Hansen AK (2008) Dietary gluten reduces the number of intestinal regulatory T cells in mice. Scandinavian journal of immunology 67: 553-559

[105] Azar ST, Tamim H, Beyhum HN, Habbal MZ, Almawi WY (1999) Type I (insulin-dependent) diabetes is a Th1- and Th2-mediated autoimmune disease. Clinical and diagnostic laboratory immunology 6: 306-310

[106] Honkanen J, Nieminen JK, Gao R, et al. (2010) IL-17 immunity in human type 1 diabetes. Journal of immunology 185: 1959-1967

[107] Flohe SB, Wasmuth HE, Kerad JB, et al. (2003) A wheat-based, diabetes-promoting diet induces a Th1-type cytokine bias in the gut of NOD mice. Cytokine 21: 149-154

[108] Lau K, Benitez P, Ardissone A, et al. (2011) Inhibition of type 1 diabetes correlated to a Lactobacillus johnsonii N6.2-mediated Th17 bias. Journal of immunology 186: 3538-3546

[109] Malaisse WJ, Courtois P, Scott FW (2004) Insulin-dependent diabetes and gut dysfunction: the BB rat model. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 36: 585-594

[110] Kaetzel CS (2014) Cooperativity among secretory IgA, the polymeric immunoglobulin receptor, and the gut microbiota promotes host-microbial mutualism. Immunology letters

[111] Sayarifard F, Aghamohammadi A, Haghi-Ashtiani MT, et al. (2012) Evaluation of serum IgA levels in Iranian patients with type 1 diabetes mellitus. Acta diabetologica 49: 131-135

[112] Satorres S, Alcaraz L, Di Genaro S (2007) Association between high levels IL-8 and Staphylococcus aureus-specific IgA antibodies in subjects with type 1 diabetes mellitus from Argentina. Diabetes research and clinical practice 77: 489-491

[113] Dunne JL, Triplett EW, Gevers D, et al. (2014) The intestinal microbiome in type 1 diabetes. Clinical and experimental immunology 177: 30-37

[114] Bekkering P, Jafri I, van Overveld FJ, Rijkers GT (2013) The intricate association between gut microbiota and development of type 1, type 2 and type 3 diabetes. Expert review of clinical immunology 9: 1031-1041

[115] Vaarala O (2013) Human intestinal microbiota and type 1 diabetes. Current diabetes reports 13: 601-607

[116] Zipris D (2013) The interplay between the gut microbiota and the immune system in the mechanism of type 1 diabetes. Current opinion in endocrinology, diabetes, and obesity 20: 265-270

[117] Hara N, Alkanani AK, Ir D, et al. (2013) The role of the intestinal microbiota in type 1 diabetes. Clinical immunology 146: 112-119

[118] Atkinson MA, Chervonsky A (2012) Does the gut microbiota have a role in type 1 diabetes? Early evidence from humans and animal models of the disease. Diabetologia 55: 2868-2877

[119] Markle JG, Frank DN, Mortin-Toth S, et al. (2013) Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science 339: 1084-1088

[120] Brugman S, Klatter FA, Visser JT, et al. (2006) Antibiotic treatment partially protects against type 1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes? Diabetologia 49: 2105-2108

[121] Hansen CH, Krych L, Nielsen DS, et al. (2012) Early life treatment with vancomycin propagates Akkermansia muciniphila and reduces diabetes incidence in the NOD mouse. Diabetologia 55: 2285-2294

[122] Sofi MH, Gudi RR, Karumuthil-Melethil S, Perez N, Johnson BM, Vasu C (2013) pH of drinking water influences the composition of gut microbiome and type 1 diabetes incidence. Diabetes

[123] Suzuki T (1987) Immune-Deficient Animals in Biomedical Research: 112-116

[124] King C, Sarvetnick N (2011) The incidence of type-1 diabetes in NOD mice is modulated by restricted flora not germ-free conditions. PloS one 6: e17049

[125] Giongo A, Gano KA, Crabb DB, et al. (2011) Toward defining the autoimmune microbiome for type 1 diabetes. The ISME journal 5: 82-91

[126] Kriegel MA, Sefik E, Hill JA, Wu HJ, Benoist C, Mathis D (2011) Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in

nonobese diabetic mice. Proceedings of the National Academy of Sciences of the United States of America 108: 11548-11553

[127] Emamaullee JA, Davis J, Merani S, et al. (2009) Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice. Diabetes 58: 1302-1311

[128] van den Brandt J, Fischer HJ, Walter L, Hunig T, Kloting I, Reichardt HM (2010) Type 1 diabetes in BioBreeding rats is critically linked to an imbalance between Th17 and regulatory T cells and an altered TCR repertoire. Journal of immunology 185: 2285-2294

[129] Bradshaw EM, Raddassi K, Elyaman W, et al. (2009) Monocytes from patients with type 1 diabetes spontaneously secrete proinflammatory cytokines inducing Th17 cells. Journal of immunology 183: 4432-4439

[130] Ferraro A, Socci C, Stabilini A, et al. (2011) Expansion of Th17 cells and functional defects in T regulatory cells are key features of the pancreatic lymph nodes in patients with type 1 diabetes. Diabetes 60: 2903-2913

[131] Schwartz RF, Neu J, Schatz D, Atkinson MA, Wasserfall C (2007) Comment on: Brugman S et al. (2006) Antibiotic treatment partially protects against type 1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes? Diabetologia 49:2105-2108. Diabetologia 50: 220-221

[132] Hageman I, Buschard K (2002) Antidiabetogenic effect of fusidic acid in diabetes prone BB rats: a sex-dependent organ accumulation of the drug is seen. Pharmacology & toxicology 91: 123-128

[133] Carlstedt-Duke B, Hoverstad T, Lingaas E, et al. (1986) Influence of antibiotics on intestinal mucin in healthy subjects. European journal of clinical microbiology 5: 634-638

[134] Calcinaro F, Dionisi S, Marinaro M, et al. (2005) Oral probiotic administration induces interleukin-10 production and prevents spontaneous autoimmune diabetes in the non-obese diabetic mouse. Diabetologia 48: 1565-1575

[135] Caballero-Franco C, Keller K, De Simone C, Chadee K (2007) The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. American journal of physiology Gastrointestinal and liver physiology 292: G315-322

[136] Schlee M, Harder J, Koten B, Stange EF, Wehkamp J, Fellermann K (2008) Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. Clinical and experimental immunology 151: 528-535

[137] Wolf KJ, Daft JG, Tanner SM, Hartmann R, Khafipour E, Lorenz RG (2014) Consumption of Acidic Water Alters the Gut Microbiome and Decreases the Risk

of Diabetes in NOD Mice. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 62: 237-250

[138] Hildebrand F, Nguyen TL, Brinkman B, et al. (2013) Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome biology 14: R4

[139] Ubeda C, Lipuma L, Gobourne A, et al. (2012) Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. The Journal of experimental medicine 209: 1445-1456

[140] Garrett WS, Lord GM, Punit S, et al. (2007) Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. Cell 131: 33-45

[141] Peng J, Narasimhan S, Marchesi JR, Benson A, Wong FS, Wen L (2014) Long term effect of gut microbiota transfer on diabetes development. Journal of autoimmunity

[142] David LA, Maurice CF, Carmody RN, et al. (2014) Diet rapidly and reproducibly alters the human gut microbiome. Nature 505: 559-563

[143] Duck LW, Walter MR, Novak J, et al. (2007) Isolation of flagellated bacteria implicated in Crohn's disease. Inflammatory bowel diseases 13: 1191-1201

[144] Ahern PP, Faith JJ, Gordon JI (2014) Mining the Human Gut Microbiota for Effector Strains that Shape the Immune System. Immunity 40: 815-823

[145] Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. Nature reviews Immunology 9: 313-323

[146] Sekirov I, Russell SL, Antunes LC, Finlay BB (2010) Gut microbiota in health and disease. Physiological reviews 90: 859-904

[147] Giongo A, Gano KA, Crabb DB, et al. (2011) Toward defining the autoimmune microbiome for type 1 diabetes. The ISME journal 5: 82-91

[148] Manichanh C, Borruel N, Casellas F, Guarner F (2012) The gut microbiota in IBD. Nature reviews Gastroenterology & hepatology 9: 599-608

APPENDIX

ANIMAL USE APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

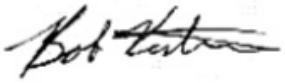
Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

Email Not Requested

DATE: November 4, 2014

TO: ROBINNA GAIL LORENZ, M.D., Ph.D.
SHEL-602
(205) 934-0676

FROM: 

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: The Gastrointestinal Ecosystem and Risk of Diabetes Development
Sponsor: Juvenile Diabetes Research Foundation International
Animal Project_Number: 140708577

As of July 25, 2014 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number In Category
Mice	A	660
Mice	B	960

Animal use must be renewed by July 24, 2015. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 140708577 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: November 4, 2014

TO: ROBINNA GAIL LORENZ, M.D., Ph.D.
SHEL-602
(205) 934-0676

FROM: Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on July 25, 2014.

Title: The Gastrointestinal Ecosystem and Risk of Diabetes Development
Sponsor: Juvenile Diabetes Research Foundation International

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).