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THE MICROBIOTA REGULATES TYPE 1 DIABETES THROUGH TOLL-LIKE

RECEPTOR SIGNALING



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# Table of Contents

# List of Figures

# List of Tables

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# Abstract

Host genetics plays an important role in shaping its intestinal microbiota. Additionally, it has been well established that the commensal microbiota is an important environmental factor that regulates autoimmunity. However, this depends on the type of autoimmune response modeled. Microbial influences range from protection, to no influence, to stimulation of disease pathology. Previous work had demonstrated that deletion of the innate immune adaptor myeloid differentiation primary response gene 88 (MyD88) in the nonobese diabetic (NOD) mouse model of type 1 diabetes (T1D) results in microbiota-dependent protection from the disease. Thus MyD88-negative mice in germfree (GF), but not in specific pathogen-free conditions, develop disease. These results could be explained by expansion of protective bacteria ("specific lineage hypothesis") or by dominance of tolerizing signaling over proinflammatory signaling ("balanced signal hypothesis") in mutant mice. We found that colonization of GF NOD with a variety of intestinal bacteria had no effect on wild-type mice, but reduced T1D in MyD88-negative NOD. These results supported the balanced signal hypothesis, but the receptors and signaling pathways involved in prevention or promotion of the disease remained unknown. The protective signals triggered by the microbiota were revealed by testing NOD mice lacking MyD88 in combination with knockouts of critical components of innate immune sensing for rescue of T1D. Only MyD88- and TIR-domain containing adapter inducing IFN β (TRIF) double deficient NOD mice developed the disease. Thus, TRIF signaling serves as one of the microbiota-induced tolerizing pathways, potentially by signaling through TLR4. TLR2 was shown to promote diabetic signaling, as the

protection observed in TLR2-negative mice was eliminated when the mice were rederived into GF conditions. Our results support the balanced signal hypothesis, in which microbes provide signals that both promote and inhibit autoimmunity. The microbiota's influence on the host immune system is regulated by activation of different members of the TLR family of innate immune receptors.

# Chapter 1: Introduction

## A brief history of diabetes

An ancient Egyptian manuscript called the *Papyrus Ebers* (1550 BCE) written by the physician Hesa-Ra makes the first known reference to a disease of "passing of too much urine" (Paul Sieving, 2014). According to ancient scholars the disease was rare and it wasn't until Araetus of Cappodocia in the first century CE that the condition received a name, diabetes (Ahmed, 2002). The word diabetes came from the Greek for "siphon" or "to pass through".

> "… no essential part of the drink is absorbed by the body while great masses of the flesh are liquefied into urine."
>
> ---Araetus of Cappodocia

Ayurveda physicians in ancient India called the disease madhumeha (honey urine), as it was noticed that patients' urine attracted ants like honey (Ahmed, 2002). By 500CE physicians in India had described two forms of diabetes, one associated with youth and another associated with weight. It wasn't until 1869 when the first piece of the underlying biology was uncovered. Paul Langerhans described a histologic examination of rabbit pancreas and noted that between the acinar glandular cells that secrete pancreatic digestive enzymes were "small cells of almost perfect homogenous content and of polygonal form…lying together in pairs or small groups" (Sakula, 1988). Although Langerhans admitted he did not know the function of the cells, he believed the unique structures signified an important function. The next important piece of the puzzle came in 1889. Minkowski and von Mering found that removal of the pancreas from dogs

resulted in the development of diabetes (Von Mering and Minkowski, 1890; Sachs, 1993). In 1893 Laguesse postulated that the cells identified by Langerhans might produce some internal excretion, and called them "Islets of Langerhans" (Goet, 1953). The next discovery was provided by Opie, where he described the hyaline degeneration of the Islet of Langerhans in diabetic patients (Opie, 1900). 9 years later two researchers independently demonstrated that secretions from the Islets of Langerhans could reduce blood sugar levels when injected into animals (De Meyer, 1909; Sharpey–Schäfer, 1914). Schaefer and de Meyer also independently coined terms for the factor, insulin (Latin for islands) (Rosenfeld, 2002). They hypothesized that the biological activity in the pancreatic secretions was a result of a single compound. Several research groups had attempted to extract the active substance from the pancreas, but contamination with pancreatic digestive proteases prevented success (Banting, 1929). The breakthrough came when Banting and Best, working with Macleod and Collip, ligated the pancreatic duct to destroy the exocrine pancreas before they attempted to extract the active substance (Banting and Best, 1922). The eventual success of the effort and subsequent clinical demonstration of efficacy has been described in great detail (Rosenfeld, 2002).

In adolescent onset diabetes life expectancy prior to the Nobel Prize winning work of Banting and Best was less than 2 years from the onset of symptoms (Joslin, 1917). By the middle of the 20th century the production of safe and effective injectable insulin transformed adolescent onset diabetes from a death sentence to a lifelong disease. With the growing use of insulin in patients it was noted that younger patients responded well to low doses of insulin while older patients were more likely to be

resistant to the drug (Falta and Boller, 1931). In 1951 Lister et al published detailed clinical observations of diabetic patients, providing a terminology for two groups of patients he observed:

> "two broad groups of diabetics—the young, thin, non-arteriosclerotic group with normal blood pressure and usually an acute onset to the disease, and the older, obese, arteriosclerotic group with hypertension and usually an insidious onset…These types we have provisionally designated type I and type II, respectively."(Lister et al., 1951)

The medical understanding of autoimmune diseases was in its infancy at this stage. In 1957 Ernst Witebsky adapted Koch postulates and proposed a set of criteria for establishing the autoimmune basis for human disease. The postulates consisted of three requirements: an autoimmune reaction identified in the form of autoantibody or cell-mediated immune reaction, the identification of the antigen targeted, and immunization of experimental animals with the antigen causes a similar disease (Witebsky, 1957; Witebsky et al., 1957). Witebsky;'s postulates and experiments in autothyroiditis spurred similar investigations into other possible diseases of "autoimmunization" (Mackay and Burnet, 1963). In Addison's disease, Grave's disease, and Hashimoto's thyroiditis monocytic infiltrates had been demonstrated in the affected tissues (Anderson et al., 1957; Roitt et al., 1956) . Additionally, self-reactive cellular immunity had been demonstrated by leukocyte migration assay (MacCuish et al., 1975; Nerup et al., 1974a). Cognizant of this paradigm, researchers examined patients with T1D. It was noted that T1D patients exhibited cellular infiltrates in their Islets of

Langerhans (Gepts, 1965; Lecompte, 1958). Nerup et al demonstrated similar activity of islet preparations to induce leukocyte migration in samples isolated from adolescent diabetics (Nerup et al., 1974a). Additional evidence that T1D was an immune-mediated syndrome came from studies linking disease susceptibility to HLA regions (Nerup et al., 1974b; Singal and Blajchman, 1973) and the identification of anti-insulin antibodies in newly diagnosed patients (Mancini et al., 1965). The immunologic basis of T1D was further demonstrated when immunosuppressive agents were shown to improve symptoms in T1D patients (Eisenbarth, 1985; Stiller et al., 1984). In a landmark paper in 1986 Eisenbarth proposed a model of the pathogenesis of T1D and described it as a chronic autoimmune disease (Eisenbarth, 1986). Thus by the late 1980s the research and medical communities understood that T1D was an autoimmune condition involving both cellular and humoral immunity and strongly linked to HLA genotype in humans.

Today we understand that T1D is a polygenic autoimmune disease, with greater than 40 genetic loci linked to disease development (Atkinson et al., 2014; Bluestone et al., 2010). The disease is characterized by destruction of the insulin producing β cells of the Islets of Langerhans in the endocrine pancreas (Jahromi and Eisenbarth, 2007). β cell death causes hyperglycemia, which if not controlled by exogenous insulin administration, results in peripheral neuropathy, cardiac complications, nephropathy, and death (National Clinical Guideline Centre (UK), 2015). T1D is an autoimmune disease afflicting over 1 million people in the US (Center for Disease Control and Prevention, 2014). This population comprises approximately 5-10% of the total diabetic population. Despite the success of insulin therapy, it is important to consider that T1D patients frequently suffer comorbidities and require lifelong insulin therapy. Insulin is not

a cure for T1D and its use is complicated by several issues: multiple insulin injections per day, measuring blood glucose more than 6 times per day, and careful monitoring of macronutrient intake. Studies show that less than 1 in 3 T1D patients achieve the target blood glucose levels set by their physicians illustrating the difficulties in managing the disease (Miller et al., 2015). Life threatening complications can arise from accumulated tissue damage due to rapid changes from healthy blood glucose concentrations to hyperglycemia or hypoglycemia. T1D patients have reduced life expectancy of 11 to 13 years and constitute a $14 billion healthcare cost in the US (Livingstone et al., 2015). Therefore, T1D represents an area of high unmet medical need for both preventative and therapeutic interventions.

## T1D and the environment

It has been documented that the incidence of T1D is increasing at a rate between 3-5% per year.  Between 2001 and 2009 there was a 21% increase in the prevalence of T1D in people under age 20 in the US (Lawrence et al., 2014). Data from European studies predict that between 2009 and 2020 the number of children diagnosed with T1D will double within the European Union.  It is important to consider that these documented increases were unlikely to reflect enhanced diagnosis of the disease. Modern diagnostic approaches and techniques are an unlikely confounding variable in the observation of increasing incidence of T1D, given the severe nature of untreated disease. Interestingly, the rate of increase differs between countries, 5% in the US, 3.9% in Europe, and 3.2% in Asia (Karvonen et al., 2000). It has been hypothesized that environmental changes must be a significant factor in the dramatic increases in disease

incidence and prevalence that have been documented.  However, the biological basis of any such environmental influences remains to be elucidated.

The best data available concerning human disease and the environment comes primarily from two approaches, twin studies and migrant studies.  It has been shown that approximately 50% of monozygotic twins are discordant for T1D (Kaprio et al., 1992; Redondo and Eisenbarth, 2002).  The interpretation being that as much as half of the risk of disease development is due to environmental factors.  However, these studies can be confounded by whether or not the twins share similar environments as well.  In an alternative approach, several studies have evaluated T1D disease development in migrant populations that have moved from a country of lower relative incidence to countries of relatively higher incidence of disease.  In studies of Asian migrant populations moving to the UK, it has been demonstrated that in one generation T1D incidence can closely reflect the new environment (Akerblom and Knip, 1998; Serrano-Rìos et al., 1999). This data supports the hypothesis that environmental factors, as well as genetics, play an important role in the development of T1D.  A variety of environmental factors have been proposed including: viral infections, childhood vaccines, and lack of breast feeding (Coppieters et al., 2012; Knip et al., 2011; Mathieu et al., 2005). Yet, at present, no agent or lifestyle practice has been conclusively demonstrated to cause disease. Interestingly, studies have demonstrated that alterations in the gut microbiota correlated with several disorders of the immune system, including T1D (Alkanani et al., 2015; Greenblum et al., 2012; Turnbaugh et al., 2007).

**The microbiota**

In discussing bacterial communities it is necessary to clearly define two key terms: the microbiome is a set of all the genes present in a microbial community, and the microbiota is a set of all microbes in a community (Hooper and Gordon, 2001; Lederberg, 2000; Whipps et al., 1988). Lederberg defined the paradigm of a superorganism, the ecological community of commensal, symbiotic, and pathogenic microorganisms associated with a host organism (Lederberg, 2000). The human microbiota consists of the 100 trillion symbiotic microbial cells harbored by each individual. The mutualistic relationship between a host and its microbiota has recently begun to be appreciated. The intestinal microbiota is a complex community that affects the health of the host by contributing to nutrition, colonization resistance to pathogenic microorganisms, and development of the immune system (Flint et al., 2012; Kamada et al., 2013; Round and Mazmanian, 2009). The human microbiome project has demonstrated that human gut commensals outnumber the number of host cells by a factor of 10, and the number of genes encoded in by microorganisms outnumber human genes by a factor of 100 (Turnbaugh et al., 2007).  These numbers serve to highlight the incredible complexity and diversity present in the ecological niches of a host organism. Thus, studies of the mammalian microbiota are complicated by the huge variability of microbial lineages that colonize a healthy host organism. An additional complicating factor is that most of the bacterial lineages have not been successfully cultured *in vitro*. This is likely to reflect the evolutionary development of co-dependence between communities of bacteria and between those communities and their host. To address these experimental limitations three approaches have proved valuable: high throughput sequencing of gut microbiota, generation of germ-free (GF) animal models,

and recolonization of GF animals with defined consortia of microbes (gnotobiotic).

Utilizing the techniques pioneered by Carl Woese, 16S rRNA gene sequences from

fecal or intestinal samples can be analyzed and classifiers assign sequences to specific

bacterial lineages (Woese and Fox, 1977). This data can establish a correlation

between a phenotype and specific lineages, but a suitable animal model is necessary to

demonstrate a causal relationship. Thus, insights into the mechanisms by which the

microbiota can influence T1D would depend on the development of a mouse model of

the disease, the non-obese diabetic (NOD) mouse.

## The NOD mouse

Up until the late 1970s animal models of T1D were limited to injection of insulin in

complete freund's adjuvant, which resulted in immune mediated destruction of the islets

in mice and rabbits (Grodsky et al., 1966). However, it was the work of Makino and

colleagues that provided a critical new tool (Makino et al., 1980). In 1966 a cataract-

prone mouse was identified from outbread ICR mice, resulting in the establishment of

the CTS strain. Selective breeding was carried out and at 20 generations a mouse that

exhibited polyuria, severe glycosuria, and weight loss was characterized (Leiter, 1993).

Breeding of the NOD mouse strain continued using offspring selected for both

spontaneous diabetes and reproductive ability.  By the 6th generation the NOD mouse

was not cataract prone, but developed diabetes at an incidence of 60-80% by 30 weeks

in female mice. Diabetes resulted from what would later be shown to be immune-

mediated destruction of the insulin producing β cells in the pancreatic islets.  The NOD

mouse has become the best studied genetically predisposed, idiopathic autoimmune animal model of human disease.

Over the following decades researchers demonstrated that this mouse model recapitulated many of the key characteristics of human disease (Pearson et al., 2015). These characteristics include common antigenic targets, expression of related peptides on MHC class II molecules, and genetic polymorphisms that affect shared disease pathways (Anderson and Bluestone, 2005). Twenty years after the linkage of HLA in humans to T1D, the MHC locus in mice was identified as the single largest contributor to disease susceptibility (Kikutani and Makino, 1992; Wicker et al., 1992, 1995). Mice have two MHC II genes, I-A and I-E, however it was demonstrated that NOD mice lacked expression of I-E (Anderson and Bluestone, 2005). Additionally, I-A in NOD mice features a non-aspartic acid substitution in the 57th position of the MHC II β chain. This finding is particularly interesting as aspartic acid in position 57 of HLA-DQ β chain is associated with resistance to T1D in humans (Cucca et al., 2001), and a non-aspartic acid substitution in this position is strongly associated with susceptibility in human populations (Horn et al., 1988; Todd et al., 1987).

In addition to the role of MHC in T1D, greater than 40 genetic loci have been identified as influencing disease development in the NOD mouse. These loci are termed insulin-dependent diabetes (*idd*) regions. Contributing loci have been investigated by genetic crosses between NOD and non-diabetic strains and given *idd* numbers. For example, the *idd5* locus contains CTLA4, a gene that has been associated with T1D in genome wide association studies of human populations (Wicker et al., 2004). Identification of overlapping genes, loci, and associated pathways between

9

human T1D and NOD supports the mouse model's value in understanding possible mechanisms in human disease. It also supports the NOD models usefulness in studying the genetic and environmental factors that influence T1D development. The NOD mouse is thought to represent a system that harbors defects in multiple tolerance and disease promoting pathways which allows T1D to occur spontaneously and stochastically. However, even without a complete understanding of the pathologic processes involved, the NOD mouse provides an opportunity to test the role of environmental factors in the development of autoimmune disease.

**NOD mice and environmental factors**

The NOD mouse strain has become an invaluable research tool in the investigation of disease mechanisms (Anderson and Bluestone, 2005) and preclinical evaluation of therapeutic interventions (Hu et al., 2007; Long et al., 2012; Reed and Herold, 2015). Importantly, research has identified that the development of disease can be modified by environmental factors. For the studies described here, we define environmental factors as any non-genetically encoded agents, whether biological, physical, or chemical. Of particular interest, biological insults include interactions between the host and pathogenic, commensal, and/or mutualistic microorganisms.

NOD mice are an ideal model to test hypotheses about environmental influences in autoimmunity. Mouse housing conditions, diet, circadian rhythm, genetics, exposure to pathogens, and the microbiota are all variables that can be controlled or manipulated to identify important variables in disease development. Initially, it was reported that female NOD diabetes incidence was 60-80% by 30 weeks, with 10-20% incidence in

males.  However it has been subsequently demonstrated that the disease incidence in both genders can vary widely between facilities and over time within the same facility (Pozzilli et al., 1993; Yurkovetskiy et al., 2013).  Researchers have identified a number of factors that influence T1D development in NOD mice, including diet and the microbiota.  It is possible that these two factors may be related, recent evidence from studies in diabetes prone BB rats identified a diet that was protective in both GF and SPF housing conditions (Patrick et al., 2013). However, it remains to be demonstrated if the protective effects of diet are dependent on the microbiota or have independent effects on T1D disease development in the NOD mouse. Additionally, as several diets have been demonstrated to alter T1D, there may be some that are dependent on the microbiota and others that are not.

In the 1980s it was documented that conventionally housed NOD mice exhibited lower diabetes incidence than those mice housed under specific pathogen free (SPF) conditions (Ohsugi and Kurosawa, 1994). Additionally, exposure of NOD mice to viral infection also reduced incidence of disease (Oldstone, 1988).  When NOD mice were rederived into germ-free conditions and observed for diabetes, these mice had an increased incidence compared with those housed in SPF conditions (Suzuki, 1987). These studies provided three interesting pieces of data: the microbiota was not required for disease development, the microbiota was associated with protection from disease, and that the gross composition of the microbiota correlated with disease phenotype.

**The microbiota and T1D**

It is well understood that environment can influence the development of autoimmunity, and more recently it has been suggested that the microbiota specifically plays a role. Humans are colonized with microbiota from their mothers during vaginal delivery. Birth by caesarian section has been demonstrated to alter the initial microbiota (Dominguez-Bello et al., 2010), and subsequently it has been observed that caesarian birth is associated with higher incidence of T1D in humans (Cardwell et al., 2008). Other studies have suggested that T1D patients and controls have differences in microbiota composition, including reduced diversity in T1D patients. However, no causative role has been defined for any species or group of microorganisms. Our best data on the effect of the microbiota in autoimmunity comes from mouse models.

Several lines of experimental evidence in mouse models support the hypothesis that specific lineages of bacteria are capable of influencing the development of autoimmunity. The presence of segmented filamentous bacteria (SFB) was shown to correlate with protection from T1D (Kriegel et al., 2011), but enhanced inflammation in a mouse model of arthritis (Wu et al., 2010). Although the underlying molecular mechanism remains unclear, the ability of SFB to induce Th17 responses has been suggested as a potential mechanism. It is thought induction of a Th17 response may function to prevent development of one disease, ex. T1D, while exacerbating the development of another, ex. arthritis. However, there are several important caveats that must be considered when interpreting the data. Recent studies have demonstrated the effect of specific lineages of bacteria can be confined within a particular context, for example monocolonization with SFB was able to protect male NOD mice but not females (Yurkovetskiy et al., 2013). Different, perhaps less well studied bacterial

lineages may have overlapping functions with the model organisms studied. For example, Altered Schaedler's Flora (ASF) is capable of inducing Th17 responses in the absence of SFB (Geuking et al., 2011). Th17 responses can be found in many animals, including humans, that do not harbor SFB (Geuking et al., 2011; Naik et al., 2012). In addition to SFB, B. fragilis has been demonstrated to be protective in animal models of colitis (Mazmanian et al., 2008). However, subsequently Clostridium spp. Clusters IV and XIVa, ASF, Bacteroides vulgatus, and Faecalibacterium prausnitzii have also demonstrated protection in colitis models (Atarashi et al., 2011; Geuking et al., 2011; Round et al., 2011). Thus some individual lineages of bacteria, such as SFB or B. fragilis, may be sufficient to induce certain disease phenotypes or immunologic parameters, but they may not be necessary to do so.

One of the most significant unaddressed questions in the microbiota field was by what mechanism(s) does the microbiota interact with the host to influence disease development. The obvious implication is that understanding how this process occurred might yield novel avenues of therapeutic approach. It would also provide an experimental framework for understanding how changes in the microbiota could influence human disease. Instead of attempting to identify specific lineages sufficient to provide the unknown autoimmune modulating signals, our approach was to disrupt the host's microbe sensing pathways. This would allow the identification of the critical pathways, and the characterization of upstream receptors and downstream effector pathways. The initial approach was the deletion of a key component of the host's ability to sense and respond to its microbiota, myeloid differentiation primary response gene 88 (MyD88) adaptor protein.

## Innate Immunity and T1D

MyD88 is a critical signaling adaptor in the innate immune response that functions downstream of members of the toll-like receptor (TLR) and IL-1 receptor families.  MyD88 provides the link between TLR family members or IL-1 receptor  to IL-1R-associated kinase (IRAK) family kinases via homotypic protein/protein interactions (Deguine and Barton, 2014).  TLR signaling via MyD88 and IRAK results in the activation of a number of transcription factors including: nuclear factor-kappa B (NFκB), mitogen-activated protein kinases, and activator protein 1. Thus MyD88 is a central adaptor of innate immunity and inflammation.

Previous work from our lab provided the first clue to unlocking the interaction between the microbiota and host signaling. NOD mice deficient in MyD88 were completely protected from T1D in SPF housing conditions (Wen et al., 2008). However, both MyD88-sufficient and MyD88-negative NOD mice rederived into germ-free (GF) conditions developed disease at a high rate of incidence (Wen et al., 2008). This data highlighted two critical points: MyD88 was not required for the disease process, and the microbiota was required for protection of MyD88-negative mice in SPF conditions.

MyD88-negative SPF NOD mice also had significant increases in several families of bacteria in their gut microbiota including: Lactobacillacae, Rikenellaceae, and Porphyromonadaceae. There was also a reduced ratio of members of the phylum Firmicutes to the phylum Bacteroidetes.  Individual bacteria or consortia of bacteria may be capable of influencing a phenotype, but demonstrating necessity is problematic. The tremendous genomic, proteomic, and metabolomic diversity in the complex

superorganism the host forms with its microbiota creates the potential for redundancy. Host organisms may rely on complex communities of microorganisms rather than individual lineages. However, the question remained were these changes in the microbiota playing a role in protection from disease? Or did the observed changes represent a different immunological environment which placed the microbiota under altered selective pressures, allowing different groups of bacteria to thrive?

**Two Hypotheses**

These questions and others formed the basis for proposing two hypotheses to explain the phenomena observed in MyD88-negative NOD mice (Chervonsky, 2013). The "specific lineage hypothesis" suggests the microbiota acquired as a neonate, and altered by the environment over time, must include specific lineages that can affect human health. Practices that alter the way we are colonized at birth, the use of antibiotics, or ecological, lifestyle, or dietary changes could all influence these specific lineages of the microbiota. These changes could affect the microbiota by reducing the population of some lineages and expanding others. To affect the development of autoimmunity in the host, these lineages must influence tolerance to host-peptides. The expanded lineages would be targeted by the mechanisms of microbial control by the host immune system. Thus there is a selective pressure on these specific lineages to suppress both innate and adaptive immune responses to maintain their expanded state, and as a by-product suppress autoimmunity.

An alternative possibility is the balanced signal hypothesis. The balanced signal hypothesis predicts that specific lineages are not required, as a level of functional

redundancy exists within the complexity of the microbiota. Specific pro or anti-inflammatory functions can be provided by multiple microbial lineages, thus the driving factor in the host-microbe relationship to disease is host genetics.  Microbes may elicit both pro and anti-inflammatory effects, and autoimmunity occurs when the host perceives these signals differently. Or alternatively, changes in the microbial communities driven by diet or other environmental factors alter the balance of pro and anti-autoimmune signals from the microbiota. The balanced signal hypothesis would explain the MyD88-negative NOD data (Wen et al., 2008) as follows; by removing MyD88 dependent pro-diabetic signaling, disease pacifying signals have been allowed to dominate. This requires that the mechanisms microbes use to pacify immune responses to microbial antigens also lead to tolerance to self-antigens.  In this context autoimmunity would be the result of the failure to induce peripheral tolerance. We had two models systems with which to test the specific lineage and the balanced signal hypotheses, the wild-type NOD mouse and the MyD88-negative NOD mouse.  We utilized SPF, gnotobiotic, and germ-free animals to dissect the role of consortia or individual species of bacteria in disease development in these two systems.

Research describing a specific role for SFB was published that demonstrated a correlation between the presence of the microorganism and protection from disease in one animal facility (Kriegel et al., 2011).  The authors identified colonization with SFB as being a strong predictor of T1D development in their NOD colony, and further associated the protection with the induction of a Th17 response in the NOD mice.  This suggested the possibility that a specific bacterium was capable of providing protection. This data supported the specific lineage hypothesis. The studies described here were

designed to address the questions raised and provide insight as to the mechanisms and pathways involved in the microbiota's regulation of T1D disease development in the NOD mouse. In chapter 1 we will examine evidence for the specific lineage hypothesis, in particular the role of SFB in the NOD mouse model.  In Chapter 2 we will address the balanced signal hypothesis and the sufficiency of a wide variety of bacteria to provide protective signals in the NOD mouse model. In chapter 3, data will be presented to support the cellular location of MyD88-dependent pro-diabetogenic signals from the microbiota.  Finally, chapter 4 will evaluate the role of reactive oxygen species generation in T1D, an effector pathway downstream of MyD88 signaling.

# Chapter 2: The specific lineage hypothesis and the limitations of the candidate bacteria approach

Colonization of the mucosal surfaces of host organisms by communities of microbes is evolutionarily conserved and is essential for survival in many host species (Eberl, 2010; Lederberg, 2000). The microbiota is also required for non-essential physiologic processes including maturation of the immune system. The question "are specific microbes required for disease processes?" has been an area of intense research in recent years.  If it was demonstrated that specific lineages were both necessary and sufficient to drive individual checkpoints in immune development, it would have dramatic therapeutic implications. In recent years Littman and colleagues have demonstrated that colonization of the small intestine with SFB was sufficient to induce CD4+ helper T cells that produce IL-17 (Th17) (Ivanov et al., 2009). They demonstrated that SFB was attached to the surface of gut epithelial cells in mice with Th17 cells (Taconic), whereas SFB was absent from mice that lacked Th17 (Jackson Labs). Colonization with SFB was correlated with expression of inflammatory and antimicrobial genes. When SFB-negative Jackson Labs C57BL/6 mice were co-housed with SFB-positive C57BL/6 mice from Taconic, mice from Jackson Labs become colonized with SFB which induce Th17 responses. The transfer of SFB and its associated inflammatory gene expression pattern enhanced resistance to a model gut pathogen, *Citrobacter rodentium*.  Thus SFB was sufficient to induce development of a protective immune response.  However, in 1974 it had been reported that SFB

heterogeneously colonized mouse research strains (Davis and Savage, 1974). This variability was demonstrated within the same strain housed at different facilities, as well as between mice within the same facility. If SFB was a specific lineage necessary for generation of proper Th17 cell populations, would variable colonization result in heterogeneous development of Th17 responses between syngeneic mice within in the same colony? Mathis and colleagues proposed that variability of SFB colonization, and thus variable induction of Th17 responses, may in part explain the stochastic nature of disease development in the NOD mouse. They surveyed their colony and confirmed variable colonization of NOD mice by SFB. Divergent colonization of mice from different animal facilities, different strains within the same facility, or different individuals within the same strain was demonstrated. Additionally, the authors showed that the presence of SFB in the gut was correlated with protection from diabetes development and insulitis. There are several possible explanations for these results including: SFB and TH17 development directly affects autoimmunity in NOD mice or SFB is associated with another microbe that induces the phenotype. It was possible that another microbe, or community of microbes, were responsible for the phenotype.

To distinguish the two possibilities we designed a series of gnotobiotic experiments. To address whether SFB alone was capable of providing protection from diabetes, GF NOD mice were gavaged with cecal contents from SFB monocolonized mice and maintained in a gnotobiotic isolator. SFB colonization was confirmed by PCR of bacterial DNA collected from fecal pellets. Once confirmed, SFB monocolonized NOD breeding pairs were established and offspring born to colonized parents were tested for SFB. Importantly, evidence of direct protection provided by SFB alone would support

the previous correlative findings that SFB provides protection from T1D in the NOD mouse. If such evidence of protection was seen in monocolonized SFB NOD mice, the data would support the specific lineage hypothesis.  All pups were confirmed colonized with SFB before weaning (Figure 1) and were observed for diabetes incidence over 30 weeks (Figure 2). At 13 (Figure 3) and 30 weeks (Figure 4) of age SFB monocolonized NOD mice were collected and pancreas tissue collected, sectioned, and scored for insulitis. We observed no reduction in diabetes incidence of SFB monocolonized NOD female mice compared with GF NOD females. However, we did observe a reduction in disease incidence in NOD male mice, those findings are presented in Yurkovetskiy et al and not reviewed here (Yurkovetskiy et al., 2013).  We observed no difference in 13 week and 30 week histology scores between SFB monocolonized NOD females and their GF counterparts.  Having established that monocultured SFB alone was not capable of protecting NOD females, we designed an experiment to test the requirement
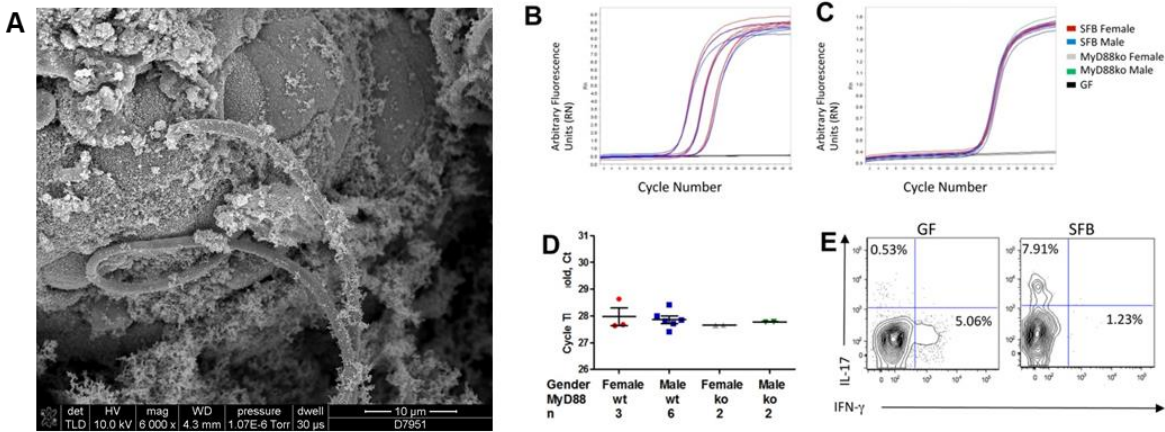
**Figure 1: Gnotobiotic colonization of GF NOD mice with SFB**

**A.** Scanning electron microscopy image of the small intestine of SFB monocolonized

NOD mice **B.** Bacterial DNA was isolated from cecal contents of SFB monocolonized

NOD mice and was analyzed by real-time PCR. Three 10-fold dilutions were compared

to ensure that the method was capable of detecting quantitative differences. Cecal

material from GF NOD mice was used as a negative control (black line) **C.** A dilution of

$10^{-3}$ was chosen for the analysis of samples isolated from SFB monocolonized NOD

males (blue lines) and females (red lines), or from MyD88ko males (green lines) and

MyD88ko females (grey lines). Cecal material from GF NOD mice was used as a

negative control (black line) **D.** Cycle threshold, Ct, values were calculated from the

amplification plots by Step-One Software (Applied Biosystems). Ct values for each

group were not statistically significantly different **E.** SFB monocolonized mice

demonstrated expansion of Th17 cells in the small intestine lamina propria of the SFB

monocolonized NOD mice compared with GF NOD mice.

21
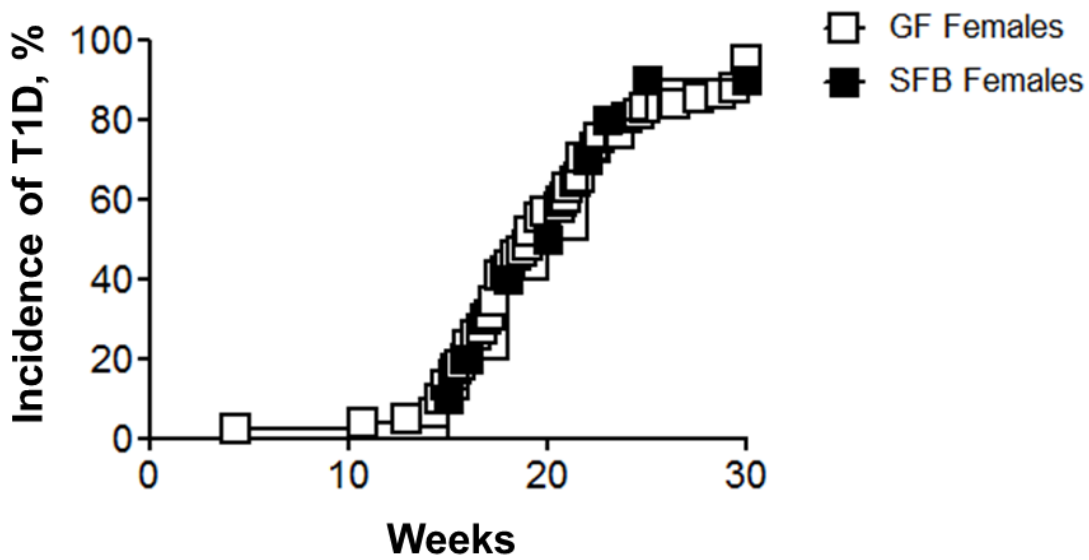
**Figure 2: Incidence of T1D observed in SFB monocolonized NOD mice**

Incidence of diabetes in SFB monocolonized female NOD mice (n=10) was compared

with that of GF female NOD mice (n=81) (P=0.645). Incidence of T1D was compared

and a P value was determined using Kaplan-Meier statistics.  n, number of animals per

group.

**Figure 3: Insulitis observed in 13 week old SPF, GF, and SFB monocolonized NOD mice**

Bona fide insulitis (percentage of total islets) in nondiabetic 13 week old female NOD mice with SPF microbiota (n=12), GF (n=15), or monocolonized with SFB (n=6). Monocolonization with SFB did not reduce the levels of insulitis in comparison to that observed in GF NOD mice ($p > 0.05$). P values for histopathology were determined by ANOVA and Bonferroni's Multiple Comparison Test. n = number of mice per group.

**Figure 4: Insulitis observed in 30 week old SPF, GF, and SFB monocolonized NOD mice**

Bona fide insulitis (percentage of total islets) in nondiabetic 30 week old female NOD mice with SPF microbiota (n=6), GF (n=6), or monocolonized with SFB (n=1). Monocolonization with SFB did not reduce the levels of insulitis in comparison to that observed in GF NOD mice (p>0.05). P values for histopathology were determined by ANOVA and Bonferroni's Multiple Comparison Test. n = number of mice per group.

for other members of the microbiota community in the presence of SFB.  We gavaged

SFB monocolonized NOD mice with cecal consents from a SFB-negative NOD mouse

from Jackson Laboratories. SFB colonization was again confirmed in the

conventionalized mice and pups were observed for diabetes incidence over a 30 week

window (Figure 5). Additionally, pancreas was collected at 30 weeks for histologic

scoring (Figure 6).  Again we saw no evidence of protection from diabetes of NOD

females colonized with SFB and SPF microbiota, as measured by disease incidence or

insulitis scores.

In our hands we found no evidence that SFB, either alone or in the presence of

Jackson Laboratories SPF microbiota, provided protection from diabetes to NOD female

mice. The apparent disparity between these resulted and the published report could be

due to several caveats of the experimental approach: SFB may interact with members

of the microbiota that are absent in the Jackson SPF microbiota, or there may be

differences in the strains of SFB used in these experiments. If the phenotype is due to

an unknown microbe that is only present at sufficient levels in the presence of SFB it

would be difficult to demonstrate with a gnotobiotic approach.  16s sequencing to

identify species or communities upregulated in the presence of SFB and subsequent

transfer of isolated populations for introduction into GF mice would be necessary.  The

lack of monocultured SFB derived from the mouse facility used by Kriegel et al makes a

functional comparison between the SFB strains difficult. Transfer of SFB-negative cecal

contents from the facility of Kriegel et al, and recolonization with each SFB strain would

be an ideal approach.  However, this is limited by technical difficulties in culturing SFB

strains *in vitro*. Our NOD mice at the University of Chicago animal facility are derived
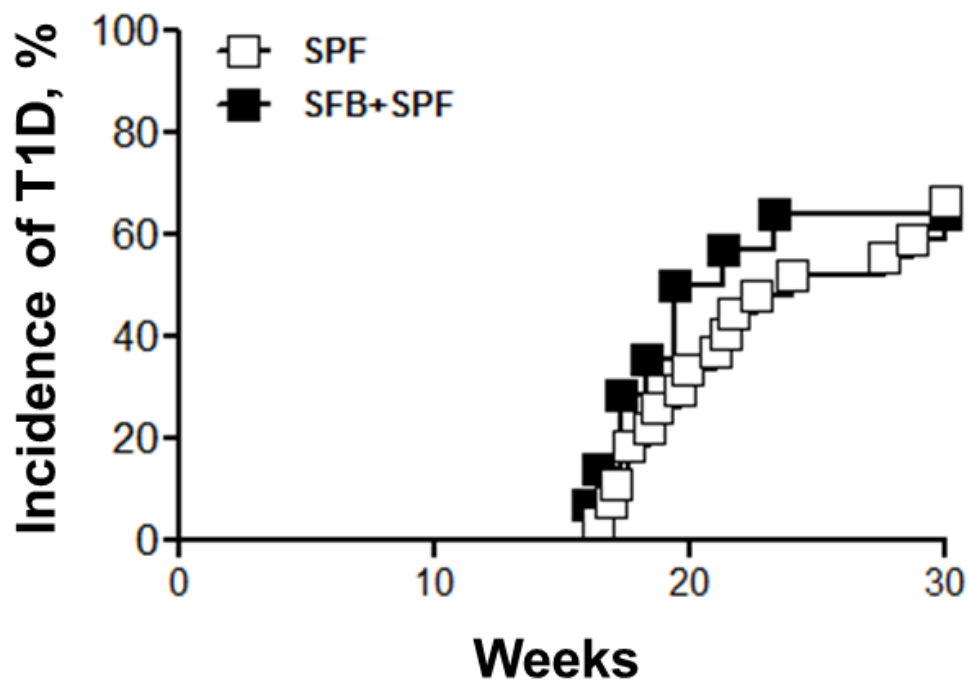
**Figure 5: Incidence of T1D observed in Jax SPF+SFB colonized NOD mice**

Incidence of diabetes in Jackson Labs SPF microbiota NOD mice (n=22) compared with Jackson Labs SPF microbiota NOD mice colonized with SFB (n=14). Colonization of Jackson Labs microbiota NOD mice with SFB did not reduce incidence of disease (p=0.6997). Incidence of T1D was compared and a p value was determined using Kaplan-Meier statistics.  n, number of animals per group.
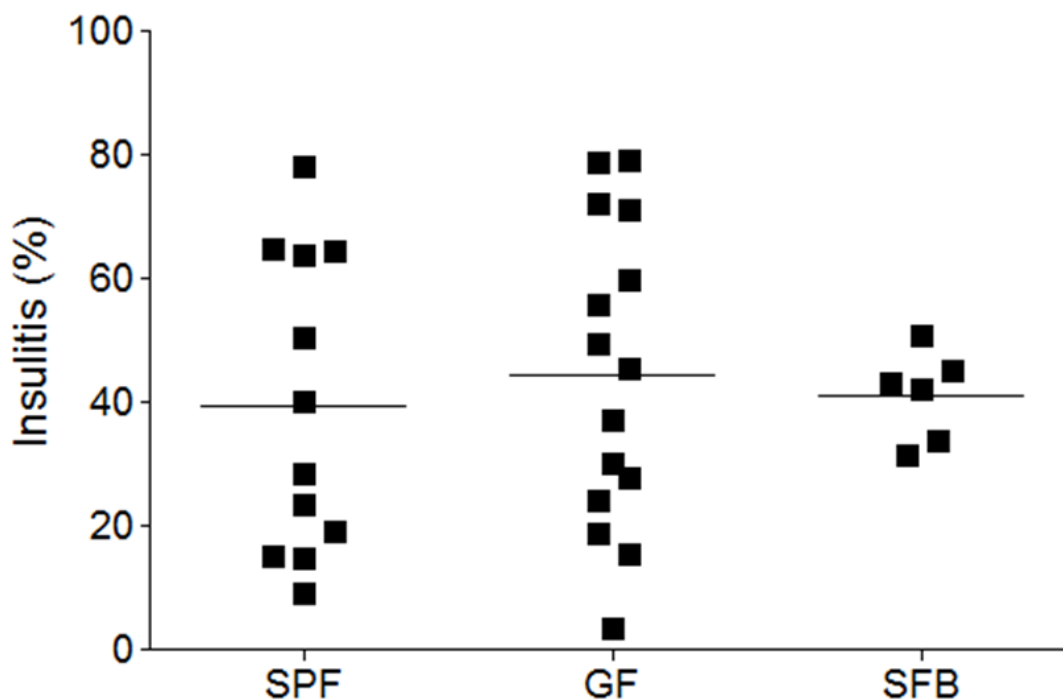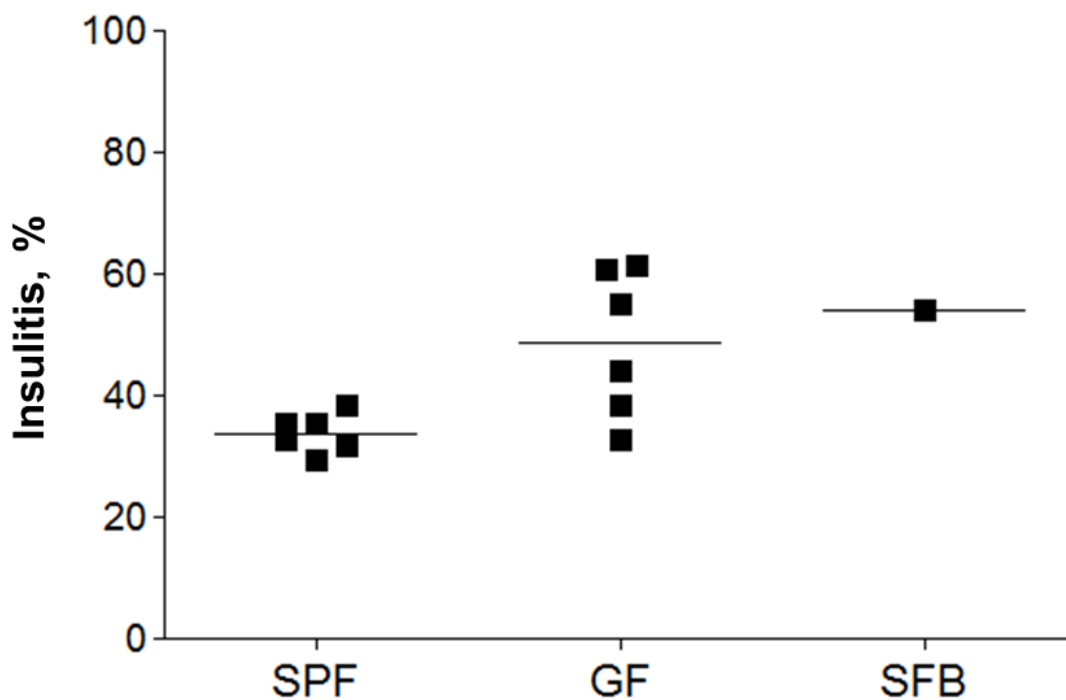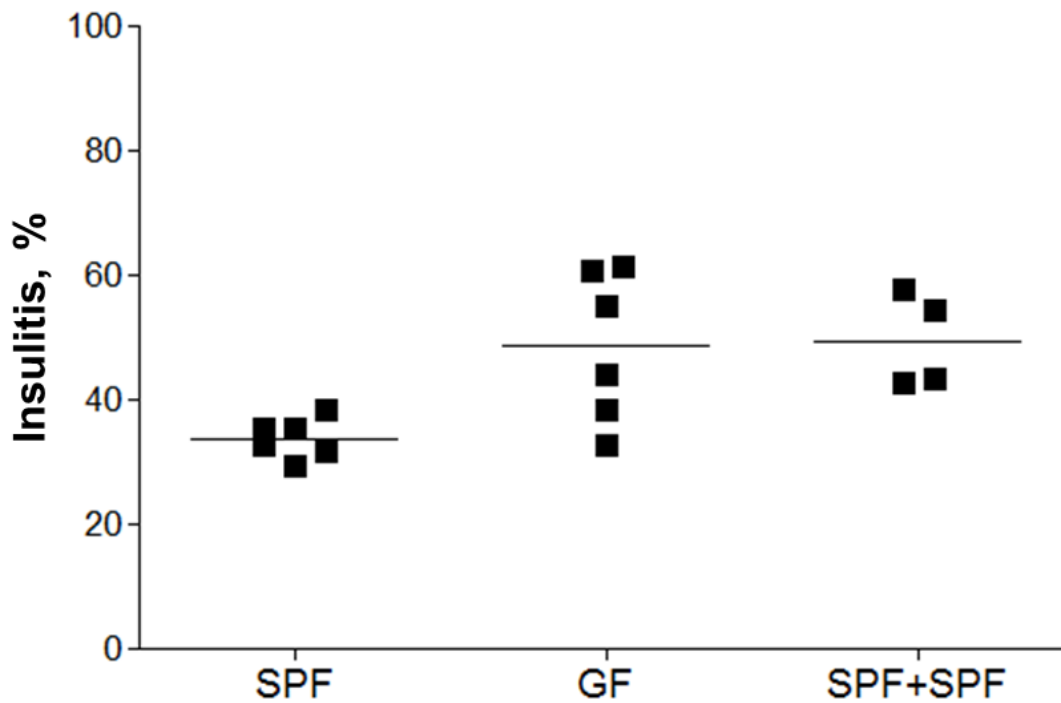
**Figure 6: Insulitis observed in 30 week old SPF and SPF+SFB NOD mice**

Bona fide insulitis (percentage of total islets) in nondiabetic 30 week old female NOD

mice with SPF microbiota (n=6), GF (n=6), or colonized with Jackson labs SPF+SFB

(n=4). Colonization with SFB in the presence of the complete Jackson labs microbiota

did not reduce the levels of insulitis in comparison to that observed in GF NOD mice

($p > 0.05$). P values for histopathology were determined by ANOVA and Bonferroni's

Multiple Comparison Test. n = number of mice per group.

from Jackson Laboratories, not Taconic, thus are not colonized with SFB. However, we observe a similar overall incidence of diabetes in SPF NOD females in our facility as reported by Kriegel et al. Additionally, other facilities have reported low incidence of T1D without the presence of SFB in the colony. We interpret these results as indicating SFB is sufficient to provide protection from disease in certain contexts, but is not required. These results highlight the difficulties in interpreting the role of specific lineages of bacteria in affecting disease phenotypes.  Organisms that provide unique signals in one facility may in fact be redundant in another facility even in syngeneic model systems. While the findings of specific roles within specific contexts may be true, this does not help us understand the underlying principles that govern the ability of the environment to regulate a phenotype. Our studies of SFB have failed to support the specific lineage hypothesis, but cannot be said to refute the hypothesis as an explanation in some contexts. These findings are a case study for the difficulty in generalizing results from candidate bacteria studies. Candidate based studies require the assumption that the bacteria directly influences the phenotype, and is not a result of interaction with other microbes.  These microbes are likely to be unknown and uncharacterized. A direct role for the candidate bacteria can only be demonstrated by monocolonization.

An alternative approach is to disrupt the pathways a host uses to sense and react to the microbiota. This approach was made possible as a result of the tremendous increase in our understanding of innate immune signaling over the past 20 years. The advantages of this approach include: identification of the critical pathway(s) regulating disease development, the ability to target the identified pathways therapeutically, the model remains agnostic as to the specific bacteria providing any signal, and the

pathways identified would provide clues as to the nature of the microbial ligand(s)

involved .The disadvantages of this approach include: pathway analysis will yield clues

as to the ligands involved but will not identify the microbes required to provide them,

host pathways may be redundant making it difficult to identify individual pathways, and

the approach is limited by the manipulations available on the correct genetic

background. With these caveats, we evaluated the ability of the microbiota to regulate

T1D in the context of genetic disruption of innate immune signaling.

## Chapter 3: Testing the balanced signal hypothesis

We had previously observed that MyD88-negative NOD mice were completely protected from T1D and that this protection was dependent on the presence of the microbiota.  Thus we proposed two potential explanations for the phenomenon: the specific lineage and the balanced signals hypotheses. In wild-type NOD mice we had failed to find evidence supporting the role of SFB as a specific lineage capable of providing protection from T1D. The balanced signal hypothesis was proposed with the understanding that the microbiota was capable of inducing immunological mechanisms that control microbes (resistance) as well as those that reduce the immune-mediated control of microbes (tolerance).  Thus we proposed that mechanisms to reduce control of microbes can also reduce autoimmune responses and yield tolerance to self.  This framework suggests microbes are providing both protective and disease promoting signals. It is the balance of these signals that is ultimately important in the regulation of autoimmunity.  It could also be suggested that given the diversity of the healthy microbiota there may be many signals provided by microbes.  The microbes capable of providing a stimulus to the host may be considerably more variable than the pathways utilized by the host to appreciate the signals.  MyD88-dependent signals represent the sum total of the disease promoting signals, given that MyD88-negative NOD mice are completely protected from diabetes in SPF conditions. Thus we asked the question, are individual microbes, or consortia of microbes, capable of providing protection from diabetes in MyD88-negative NOD mice?

30

To address this question we colonized GF NOD MyD88-heterozygous or MyD88-negative littermates with two consortia of bacteria (ASF and VSL3) and one individual species (SFB).  We collected pancreas from gnotobiotic NOD mice at 13 weeks to score the levels of insulitis in the Islets of Langerhans. We had previously demonstrated that the percentage of insulitis at this time point strongly correlated with overall disease incidence (Yurkovetskiy et al., 2013). Additionally, we had demonstrated that ASF colonized gnotobiotic MyD88-negative NOD mice were significantly protected from diabetes (Wen et al., 2008). ASF is a consortium of eight mouse commensal microbes, and as expected colonization was sufficient to reduce insulitis in MyD88-negative NOD mice (Figure 7). Thus, a limited group of healthy mouse commensals largely recapitulated the phenotype of NOD MYD88-negative mice harboring SPF microbiota. Interestingly we also saw significant levels of protection in VSL3 colonized NOD MyD88-negative mice.  VSL3 is a human probiotic mix of three *Bifidobacteria*, four *Lactobacilli*, and one *Streptococcus*. Thus a consortium of evolutionarily adapted human gut microbes were also sufficient to provide the protective signals in MyD88-negative NOD mice.  Finally, monocolonization with SFB significantly reduced insulitis at 13 weeks in MyD88-negative NOD mice. This demonstrated that a single strain of bacteria

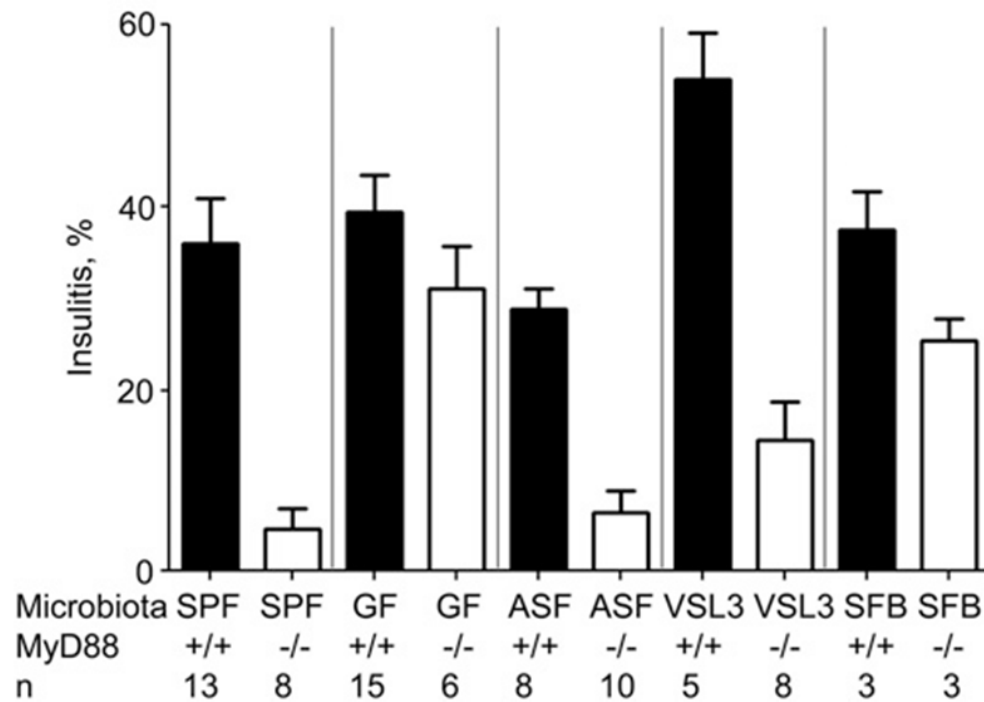**Figure 7: Distinct bacterial lineages reduce diabetes incidence in gnotobiotic MyD88-negative NOD mice.**

Bona fide insulitis (percentage of total islets) in 13-wk-old female MyD88+/+ or MyD88-negative mice in SPF and GF conditions and colonized with ASF, VSL3, or SFB. n = number of mice per group. P values for histopathology were determined by Student's t test. *P < 0.05, **P < 0.01, ***P < 0.001.

was capable of providing partial protection in the absence of MyD88. We concluded that the protective signals that provided protection in MyD88-negative NOD mice could be provided by a wide range of microbes. The broad variety of microbes capable of providing anti-diabetic signaling supported the balanced signal hypothesis. Organisms not evolutionarily adapted to the mouse gut, or even a single bacterium, was sufficient to provide the unknown MyD88-independent protective signal(s). However, it must be pointed out that while the levels of insulitis were reduced in these cohorts, gnotobiotic colonization did not provide 100% protection, as afforded by the entire SPF microbiota. This could be interpreted as either the levels of the unknown signaling agent were reduced in the gnotobiotic NOD mice, or that multiple ligands and pathways contribute to the prevention of diabetes in MyD88-negative NOD mice.

As described previously, the variety of microbes capable of providing these protective signals in the absence of MyD88 may be myriad. We reasoned that a productive approach would be to attempt to identify the pathway(s) that the host uses to sense and respond to the microbiota. We hypothesized that one or more pattern recognition receptors (PRR) utilized by the innate immune system was a likely candidate, given the role of MyD88 in disease promoting signals. Additionally, identifying the innate immune sensors or effectors involved would narrow the spectrum of microbial ligands capable of providing the signal. We focused our efforts on the NOD-like receptors (NLR), the inflammasome, and MyD88-independent TLR signaling. Initially we needed to demonstrate if deletion of any of these elements of the immune system directly affected diabetes incidence in NOD mice.

The inflammasome is a complex of signaling molecules whose oligomerization activates caspase 1 and leads to the production of proinflammatory effectors such as IL-1 and IL-18. Oligomerization occurs following activation by a variety of ligands, including some of bacterial origin. IL-1 and IL-18 signaling had previously been demonstrated to be dispensable in the induction of T1D in NOD mice, as Caspase 1-negative NOD mice had the same incidence of diabetes as their heterozygous littermates. (Schott et al., 2004). Additionally, both IL-1 and IL-18 signal via MyD88, and thus could not provide the protective signal in MyD88-negative NOD mice.  However, the caspase 1-negative mouse has been shown to lack expression of caspase 11 as well.  Caspase 4/11 oligomers have been shown to provide innate immune signaling by binding to LPS. Caspase 4/11 represents an additional MyD88-independent innate immune signaling pathway; therefore we observed the incidence of T1D in NOD Caspase 1/11-negative NOD mice in our facility (Figure 8). Deletion of Caspase 1/11 did not affect diabetes incidence, indicating that it does not directly influence disease.

The NLR family is a group of intracellular receptors. NOD1 and NOD2 recognize the bacterial cell wall component peptidoglycan and ligand-bound NOD1/2 oligomerize and signal through the serine-threonine RIP2 kinase via CARD–CARD homophilic interactions (Kobayashi et al., 2002). Activated RIP2 directs the ubiquitination of NEMO/IKKγ, which induces activation of NF-κB and inflammatory cytokine release. RIP2-negative C57BL/6 mice were backcrossed to NOD for more than 10 generations. To test for a direct role of NLRs in T1D we observed diabetes incidence in RIP2-heterozygous and RIP2-negative littermates (Figure 9). The genetic elimination of NLR

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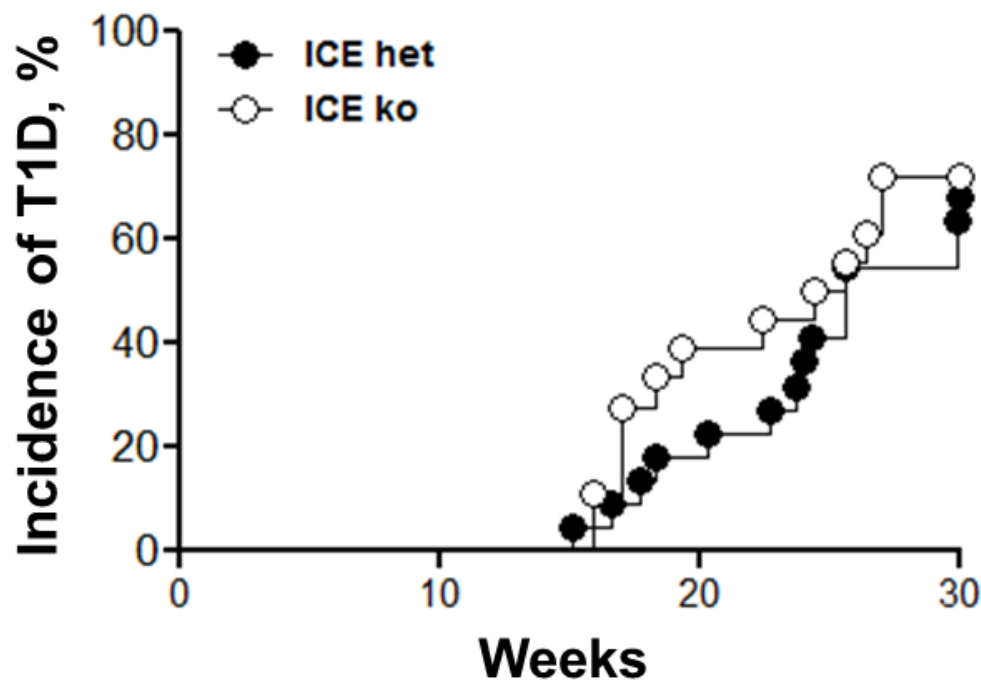
**Figure 8: Ablation of Caspase 1/11 signaling does not prevent T1D**

Diabetes incidence in NOD female caspase 1/11-heterozygous (n=22), caspase 1/11-negative (n=18) littermates. Removal of signaling via Caspase 1/11 did not reduce incidence of disease compared with their heterozygous littermates (p=0.525). P value was determined using Kaplan-Meier statistics.  n, number of animals per group.

**Figure 9: Ablation of NLR signaling does not prevent T1D**

Diabetes incidence in NOD female RIP2-heterozygous (n=12), RIP2-negative (n=14) littermates. Removal of NLR signaling via deletion of RIP2 did not reduce incidence of disease compared with their heterozygous littermates (p=0.375). P value was determined using Kaplan-Meier statistics.  n, number of animals per group.

signaling had no influence on T1D as both RIP2-heterozygous and RIP2-negative littermates developed diabetes at a similar incidence.

TLRs utilize two signaling adaptor molecules, MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) encoded by the gene *ticam1*. Several studies have demonstrated a role for TRIF as a negative regulator of immunity (Baetz et al., 2004; Rathinam et al., 2012; Seregin et al., 2011). Thus we backcrossed C57BL/6 TRIF-negative mice to NOD for more than 10 generations and observed for diabetes incidence over 30 weeks (Figure 10).  No reduction in T1D incidence was observed in TRIF-negative NOD mice compared with their TRIF-heterozygous littermates.  We concluded that TRIF, as with Caspase 1/11 and RIP2, was a viable candidate pathway for providing the microbiota-dependent, MyD88-independent protective signal(s).

To test the role of these innate immune sensors in providing the MyD88-independent, microbiota-dependent signal we designed a complementation experiment. Knockouts of an innate sensing pathway would be crossed to MyD88-negative NOD mice to generate littermates who both lacked MyD88 and were either heterozygous or a knockout for the pathway of interest.  We hypothesized that removal of any pathway not required to provide a protective signal would result in complete protection, as seen in the MyD88-negative alone.  However, if a pathway was required to provide some proportion of the protective signal its removal would result in a rescue of diabetes incidence. This approach would identify individual pathways that were required for protection. This approach would also demonstrate how much any pathway contributed to the overall level of protection. This approach allowed for the identification of critical microbiota-dependent protective pathways utilized by the host, and provided clues as to
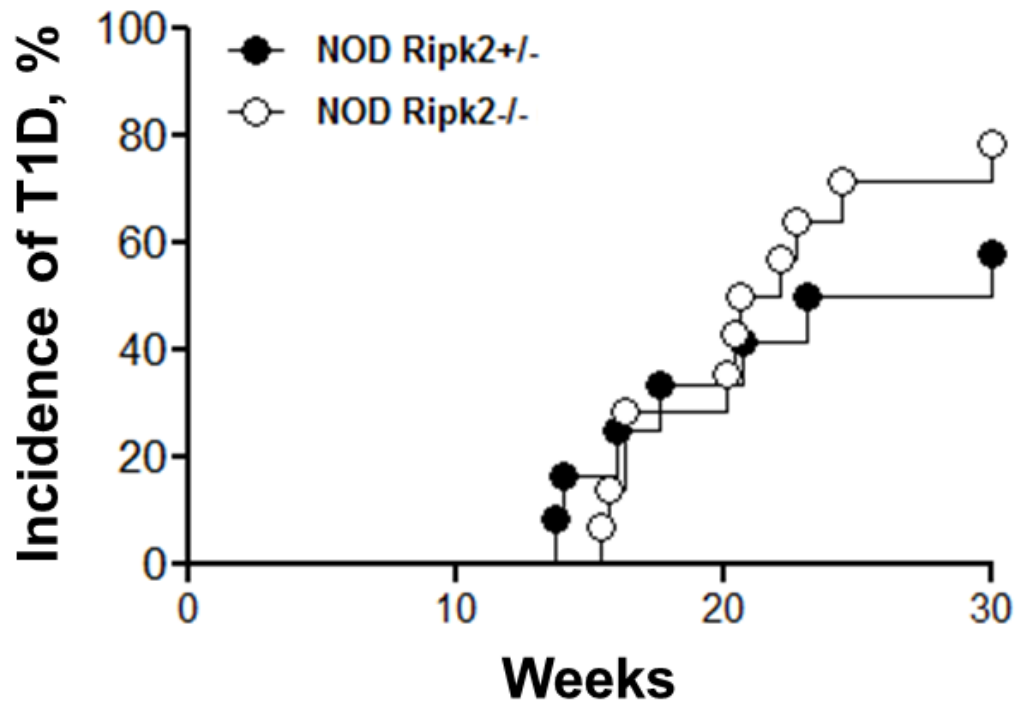
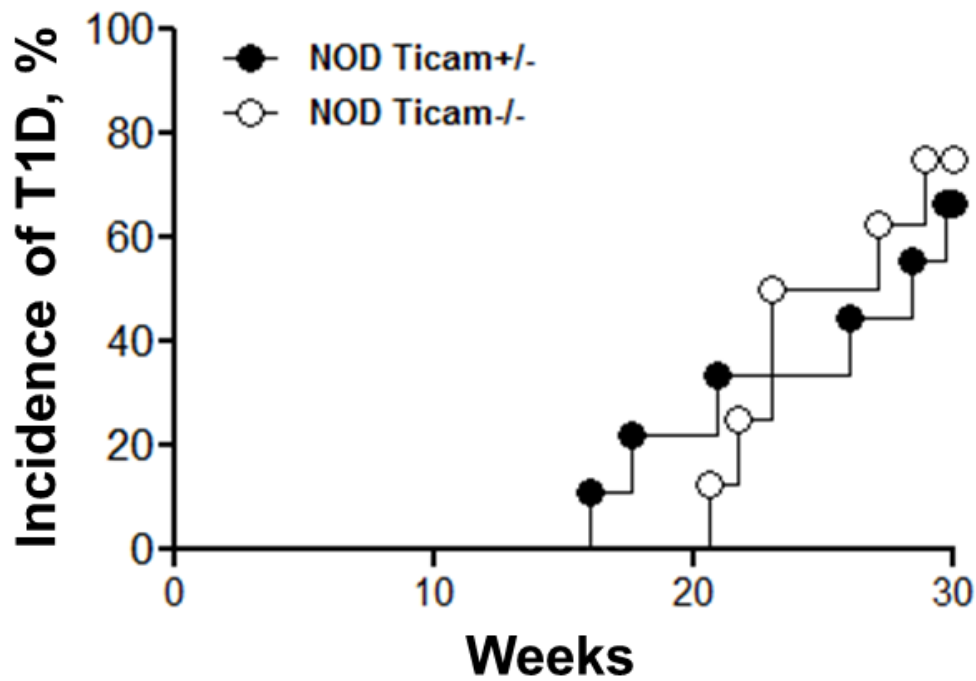**Figure 10: Ablation of TRIF signaling does not prevent T1D**

Diabetes incidence in NOD female *Ticam1*-heterozygous (n=9), *Ticam1*-negative (n=8)

littermates. Removal of TRIF signaling via disruption of *Ticam1* did not reduce incidence

of disease compared with their heterozygous littermates (P=0.779). P value was

determined using Kaplan-Meier statistics.  n, number of animals per group.

what the potential ligands that generated the protective signals were. However, if the protective pathways were functionally redundant, knockouts of multiple MyD88-independent pathways in the same NOD mouse would need to be made and tested.

To test the role of Caspase 11 in providing protection from diabetes in the absence of MyD88, we crossed Caspase1/11-negative NOD mice to MyD88-negative mice and generated NOD MYD88-negative Caspase 1/11-negative and NOD MyD88-negative Caspase 1/11-heterozygous littermates for observation of disease incidence (Figure 11). Pancreas tissue was collected at 30 weeks of age and histologic scoring of insulitis was performed (Figure 12). The deletion of caspase 1/11 did not rescue T1D as the double deficient NOD mice remained protected from disease. This indicated the LPS-caspase 11 innate immune pathway did not provide microbiota-dependent protection in the absence of MyD88.

To test the role of NLRs in providing protection from diabetes in the absence of MyD88, we crossed RIP2-negative NOD mice to MyD88-negative mice and generated NOD MYD88-negative RIP2-negative and NOD MyD88-negative RIP2-heterozygous littermates for observation of disease incidence (Figure 13). Pancreas tissue was collected at 30 weeks of age and histologic scoring of insulitis was performed (Figure 14). The deletion of RIP2, and thus NLR signaling, did not rescue T1D as the double deficient NOD mice remained protected from disease. Thus the NLR innate immune pathway did not provide microbiota-dependent protection in the absence of MyD88.

To test the role of MyD88-independent TLR signaling in providing protection from diabetes in the absence of MyD88, we crossed TRIF-negative NOD mice to MyD88-negative mice. NOD MYD88-negative TRIF-negative and NOD MyD88-negative TRIF-
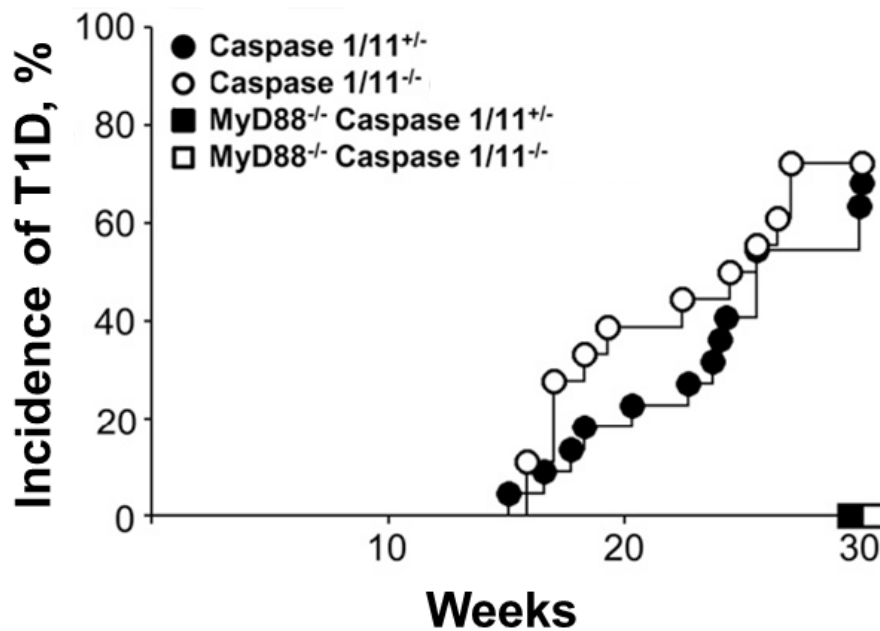
**Figure 11: Ablation of Caspase 1/11 signaling in the absence of MyD88 fails to rescue T1D.**

Diabetes incidence in NOD female caspase 1/11-heterozygous (n=22), caspase 1/11-negative (n=18) littermates, and MyD88-negative caspase 1/11-heterozygous (n=18), MyD88-negative caspase 1/11-negative (n=9) littermates. Removal of signaling via Caspase 1/11 in the absence of MyD88 did not rescue disease development compared with their heterozygous littermates (P>0.05). P value was determined using Kaplan-Meier statistics.  n, number of animals per group.
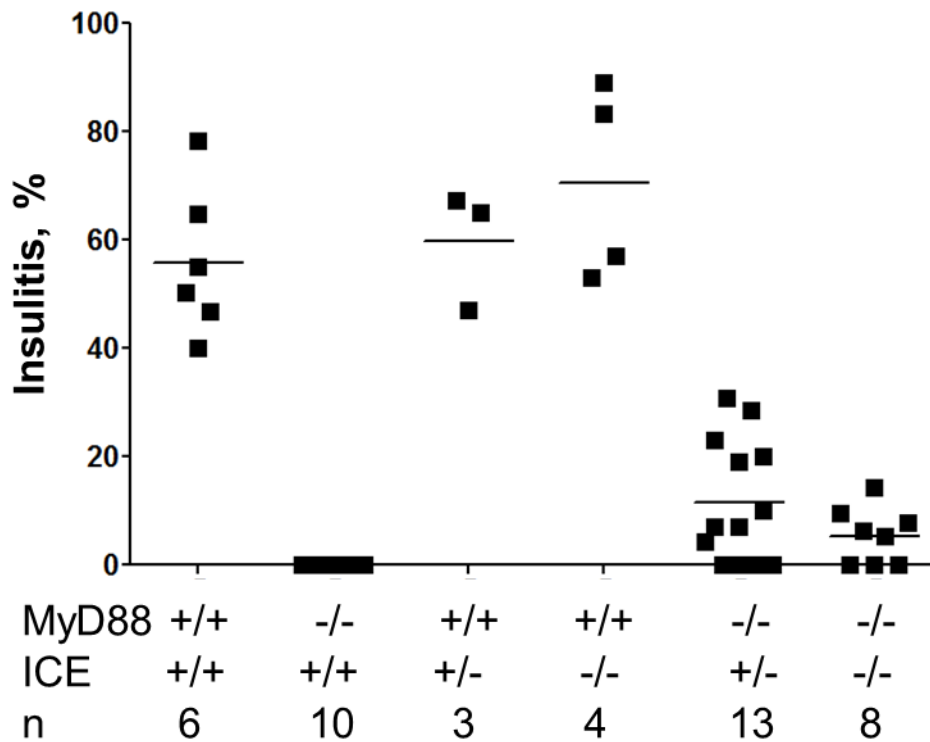
**Figure 12: Insulitis observed in 30 week old MyD88-negative ICE-negative NOD mice**

Bona fide insulitis (percentage of total islets) in nondiabetic 30 week old female ICE-heterozygous, ICE-negative littermates, and MyD88-negative ICE-heterozygous, MyD88-negative ICE-negative littermates. Deletion of Caspase 1/11 signaling in the absence of MyD88 did not rescue insulitis in double knockout NOD mice compared with their Caspase 1/11-heterozygous littermates ($p > 0.05$). P values for histopathology were determined by ANOVA and Bonferroni's Multiple Comparison Test. n = number of mice per group.

**Figure 13: Ablation of NLR signaling in the absence of MyD88 fails to rescue T1D**

Diabetes incidence in NOD female RIP2-heterozygous (n=12), RIP2-negative (n=14) littermates, and MyD88-negative RIP2-heterozygous (n=8), MyD88-negative RIP2-negative (n=3) littermates. Removal of NLR signaling by deletion of RIP2 in the absence of MyD88 did not rescue disease development compared with their heterozygous littermates (P>0.05). P value was determined using Kaplan-Meier statistics.  n, number of animals per group.
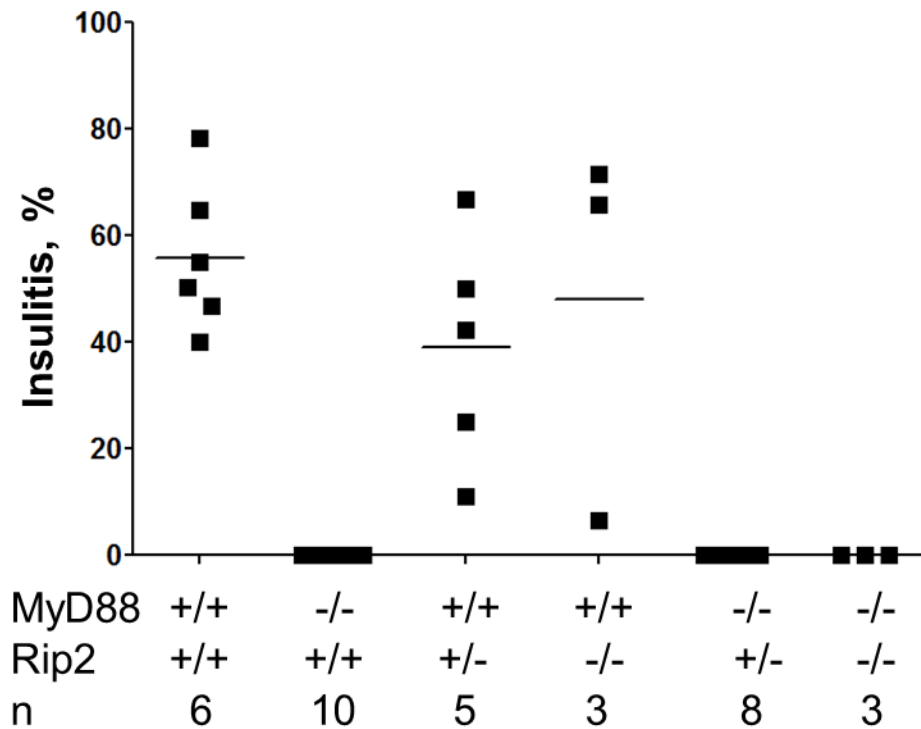
**Figure 14: Insulitis observed in 30 week old MyD88-negative RIP2-negative NOD mice**

Bona fide insulitis (percentage of total islets) in nondiabetic 30 week old female RIP2-heterozygous, RIP2-negative littermates, and MyD88-negative RIP2-heterozygous, MyD88-negative RIP2-negative littermates. Deletion of RIP2 signaling in the absence of MyD88 did not rescue insulitis in double knockout NOD mice compared with their RIP2-heterozygous littermates ($p > 0.05$). P values for histopathology were determined by ANOVA and Bonferroni's Multiple Comparison Test. n = number of mice per group.

heterozygous littermates were generated for observation of disease incidence (Figure 15). Pancreas tissue was collected at 30 weeks of age and histologic scoring of insulitis was performed (Figure 16). The deletion of TRIF rescued T1D as the double deficient NOD mice developed T1D. In the University of Chicago animal facilities MyD88-negative NOD mice exhibit 100% protection from T1D, thus the reversal (although partial) was both biologically and statistically significant. This conclusion was further strengthened by the histologic analysis of insulitis at 30 weeks of age. A significantly higher amount of islet immune infiltration was observed in double knockout NOD mice compared with MyD88-negative TRIF-heterozygous NOD mice. Thus, even those double deficient NOD mice that did not become diabetic within 30 weeks exhibited a higher proportion of insulitis. This data supported the conclusion that signaling by TRIF, potentially through TLR3 or TLR4, contributed to protection from T1D in MyD88-negative NOD mice.

## Chapter 4: MyD88-dependent signaling in T1D

Having identified the TLR adaptor molecule TRIF as an important factor in anti-diabetic signaling, we focused on the role of MyD88. MyD88-dependent signals provided 100% of the microbiota-dependent diabetes promoting signal. We hypothesized that these disease driving signals were a result of activation of TLRs upstream of MyD88. We excluded a role for MyD88 signaling through IL-1R, as caspase 1-negative mice were demonstrated to efficiently develop T1D (Schott et al., 2004). Thus MyD88-dependent signals from the microbiota were a result of activation by TLRs. In mice TLRs 1, 2, 5, 6, 7, 9, and 11 exclusively utilize MyD88 for signaling, TLR 3 exclusively utilizes TRIF, and TRL4 uses both signal adaptors. Previous studies had demonstrated that genetic ablation of a variety of TLRs resulted in different effects on T1D incidence in the presence of microbes. Individual removal of TLR 2 (Kim et al., 2011; Wen et al., 2008) and TLR 9 (Wong et al., 2008) resulted in partial reduction of diabetes incidence. There are two possible explanations for the observed phenomena: direct contribution of the TLR to development of T1D, or the TLR is responsible for a proportion of the MyD88-dependent, microbiota-dependent signal that drive autoimmunity in T1D. To differentiate these two possibilities we rederived TLR2-negative NOD mice into GF housing conditions and observed the development of T1D (Figure 17). As expected, in SPF housing conditions TLR2-negative NOD mice exhibited a reduced incidence of T1D compared with their TLR2-heterozygous littermates (approximately a 50% reduction). Interestingly, GF TLR2-negative NOD mice and their TLR2-heterozygous littermates both developed T1D at a high rate of incidence
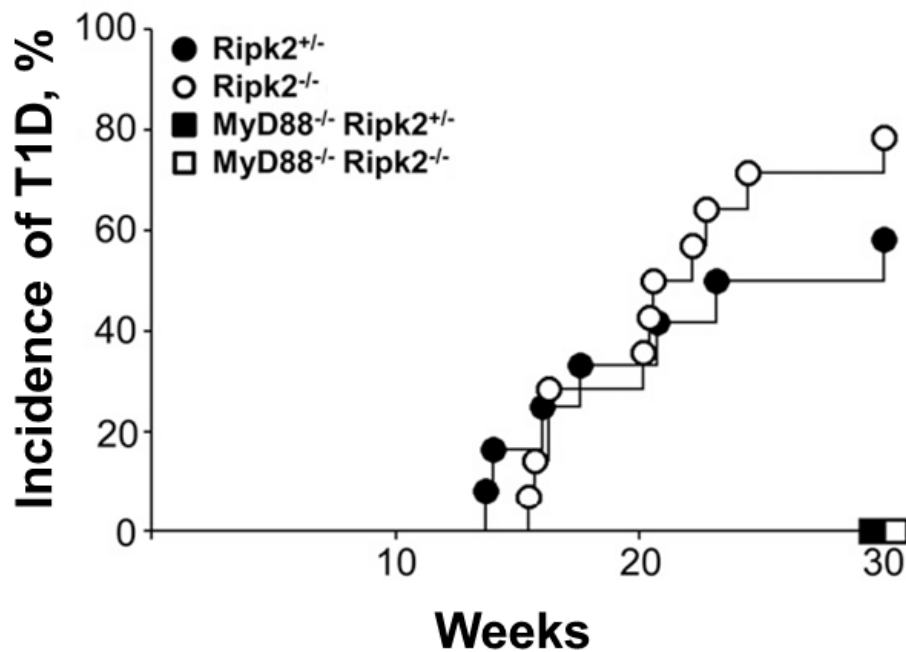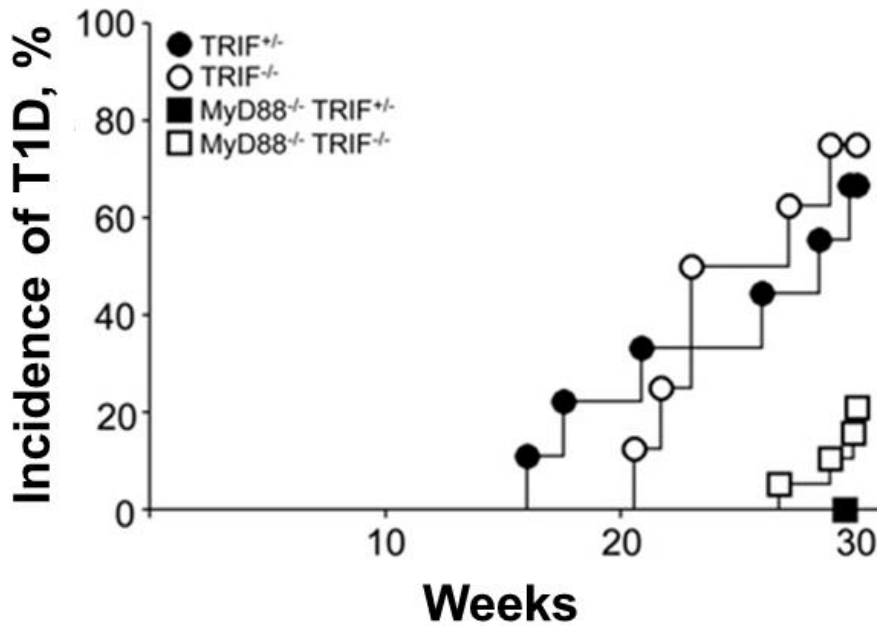
**Figure 15: Ablation of TRIF signaling in the absence of MyD88 rescues T1D**

Diabetes incidence in NOD female *Ticam1*-heterozygous (n=9), *Ticam1*-negative (n=8)

littermates, and MyD88-negative *Ticam1*-heterozygous (n=12), MyD88-negative

*Ticam1*-negative (n=19) littermates. Removal of TRIF signaling by deletion of *Ticam1* in

the absence of MyD88 rescued disease development compared with their heterozygous

littermates (p=0.0325). P value was determined using Kaplan-Meier statistics.  n,

number of animals per group.
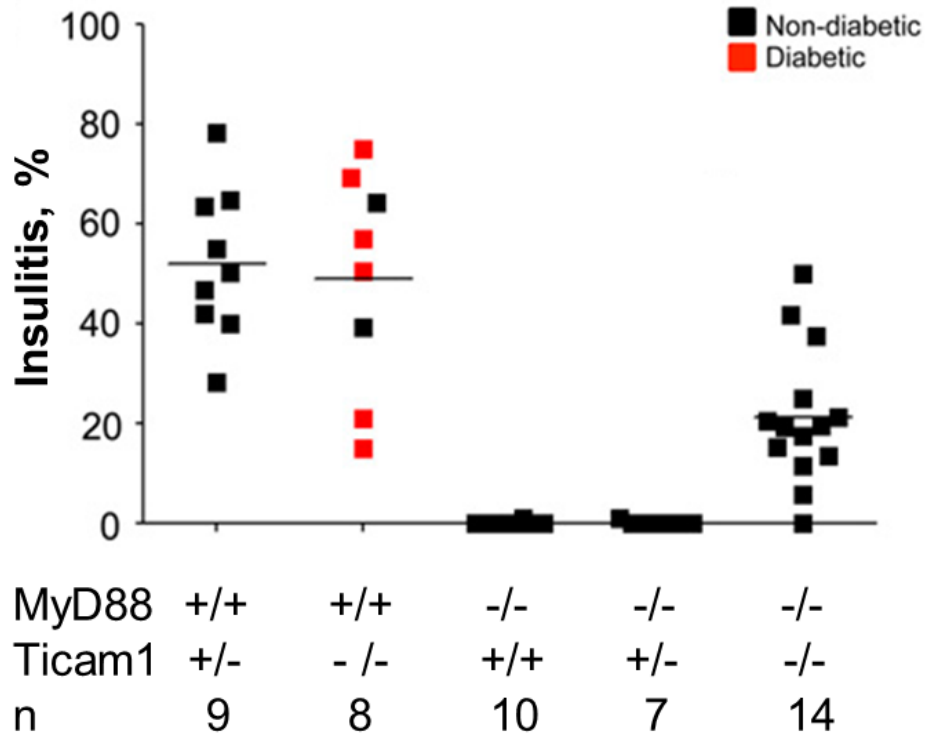
**Figure 16: Insulitis observed in 30 week old MyD88-negative TRIF-negative NOD mice**

Bona fide insulitis (percentage of total islets) in diabetic (red) and nondiabetic (black) 30 week old female *Ticam1*-heterozygous, *Ticam1*-negative littermates, and MyD88-negative *Ticam1*-heterozygous, MyD88-negative *Ticam1*-negative littermates. Deletion of TRIF signaling in the absence of MyD88 rescued insulitis in double knockout NOD mice compared with their *Ticam1*-heterozygous littermates (p>0.05). P values for histopathology were determined by ANOVA and Bonferroni's Multiple Comparison Test. n = number of mice per group.

and had increased histopathology scores compared with SPF TLR2-negative mice (Figure 18). Therefore we concluded that the reduced incidence of T1D in TLR2-negative NOD mice was an indirect effect. The knockout of TLR2 had allowed the microbiota to reduce the autoimmune response. TLR2 was a receptor upstream of MyD88, providing part of the microbiota-dependent signal by signaling through MyD88. Previous studies had demonstrated that deletion of TLR3 had no effect on T1D in NOD mice (Wong et al., 2008). However, ablation of TLR4 enhanced the development of T1D. It was observed in TLR4-negative NOD mice that the total incidence was higher and the rate of disease onset was accelerated compared with TLR4-heterzygous littermates (Dong et al., 2012; Gülden et al., 2013). We hypothesized that these findings were a result of TLR4 activating a MyD88-independent signaling pathway that promoted tolerance to self (i.e. TRIF). To test if TLR4 signaling promoted tolerance to self in a microbiota-dependent manner, we rederived TLR4-negative NOD mice into GF housing conditions. We observed diabetes incidence over 30 weeks in both GF and SPF conditions (Figure 19). We also collected pancreas for histologic scoring of islet infiltration at 30 weeks, or at the time of diabetes onset (Figure 20). As expected, SPF TLR4-negative NOD mice exhibited some acceleration of disease onset. Interestingly, in GF conditions TLR4-negative and their TLR4-heterozygous littermates developed disease at the same rate. We concluded that microbiota-induced TLR4 activation is a protective signal to reduce T1D development in NOD mice. Together this data suggested that TLR2 activation upstream of MyD88 promoted T1D, whereas TLR4 activation upstream of TRIF protected from disease in SPF NOD mice.
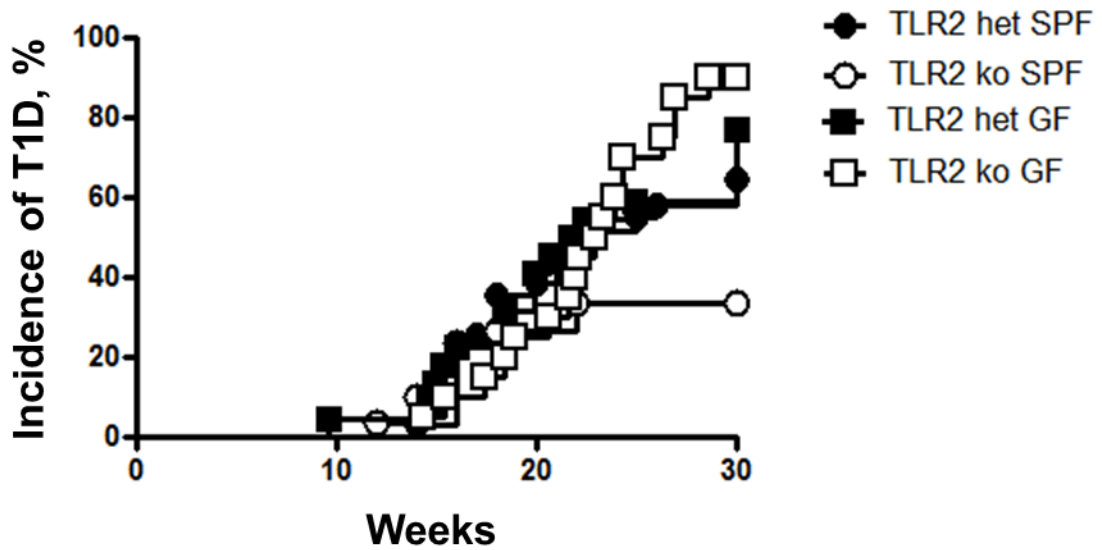
**Figure 17: Deletion of TLR2 protects in SPF, but not GF conditions**

Diabetes incidence in SPF NOD female TLR2-heterozygous (n=31), TLR2-negative

(n=30) littermates, and GF NOD female TLR2-heterozygous (n=22), TLR2-negative

(n=20) littermates. Removal of TLR2 signaling in SPF, but not in GF conditions, reduced

disease development compared with their heterozygous littermates (p=0.007). P value

was determined using Kaplan-Meier statistics.  n, number of animals per group.

**Figure 18: Insulitis observed in 30 week old TLR2-negative SPF and GF NOD mice**

Bona fide insulitis (percentage of total islets) in diabetic (red) and nondiabetic (black) 30 week old female SPF TLR2-heterozygous, TLR2-negative littermates, and GF TLR2-heterozygous, TLR2-negative littermates. Deletion of TLR2 signaling in in SPF, but not in GF conditions (p=0.8959), reduced disease development compared with their heterozygous littermates (p=0.0039). P values for histopathology were determined by Student's *t* test. n = number of mice per group.

Having identified TLR2 as a key receptor upstream of MyD88, we wanted to elucidate the cellular location of the MyD88 phenotype. It had been conclusively demonstrated the deletion of MyD88 in all mouse cells efficiently protected from disease. We proposed that deletion of MyD88 in individual cell subsets would reveal important aspects of the underlying biology of T1D. To approach this task we used a variety of tools including bone marrow chimeras and promoter specific Cre recombinase driven deletion of MyD88 alleles flanked by LoxP sites. The Cre/Lox approach was limited by the cell specific promoter-Cre mice available on the NOD background. MyD88-negative mice do not survive lethal irradiation for reconstitution of bone marrow. Additionally, it is well documented that NOD mouse bone marrow is radioresistant (Anderson and Bluestone, 2005). Recent studies utilizing a NOD congenic strain allowed the identification of the resistant populations in NOD mice (Steptoe et al., 2004). The authors described efficient repopulation of myeloid derived cells including macrophages, dendritic cells, and neutrophils. However, T lymphocyte progenitors were incompletely eliminated following superlethal irradiation (1200 rads). Thus the traditional bone marrow chimera approach to segregate the phenotype by hematopoietic and stromal compartments was not possible.

As an alternative approach we acquired a NOD mouse with a congenic marker from Jackson Laboratories. The NOD CD45.2 congenic strain contains *Ptprc* of C57BL/6J origin, which replaced the NOD CD45.1 allele. This strain was shown to exhibit high incidence of T1D at Jackson Laboratories. This tool allowed us to transfer bone marrow from NOD CD45.1 MyD88-heterozygous or MyD88-negative NOD mice into NOD CD45.2 recipients. Using the congenic markers we were able to distinguish
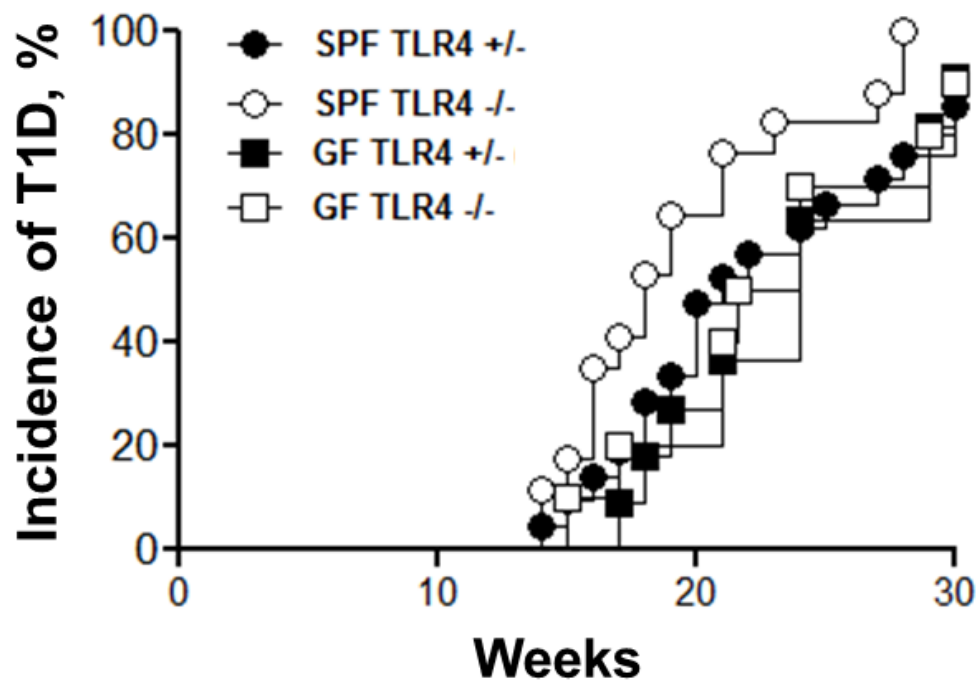
**Figure 19: Deletion of TLR4 accelerates T1D incidence in SPF but not GF conditions**

Diabetes incidence in SPF NOD female TLR4-heterozygous (n=21), TLR4-negative (n=17) littermates, and GF NOD female TLR4-heterozygous (n=1), TLR4-negative (n=10) littermates. Removal of TLR4 signaling in SPF, but not in GF conditions, accelerated disease development compared with their heterozygous littermates (P=0.0371). P value was determined using Kaplan-Meier statistics.  n, number of animals per group.
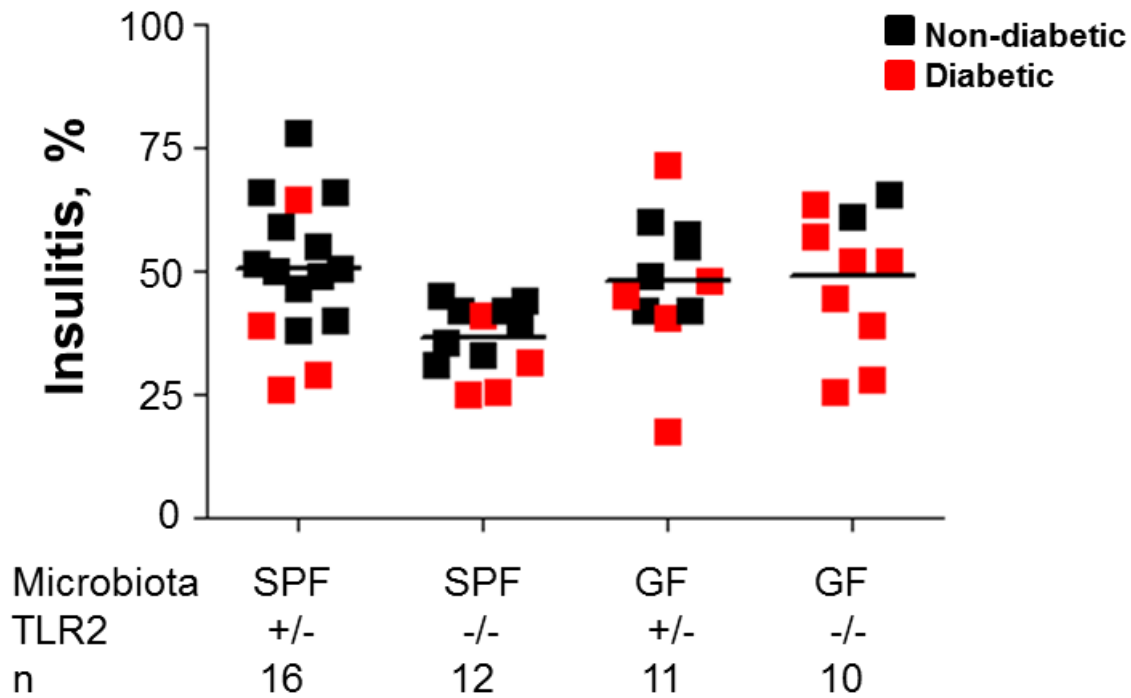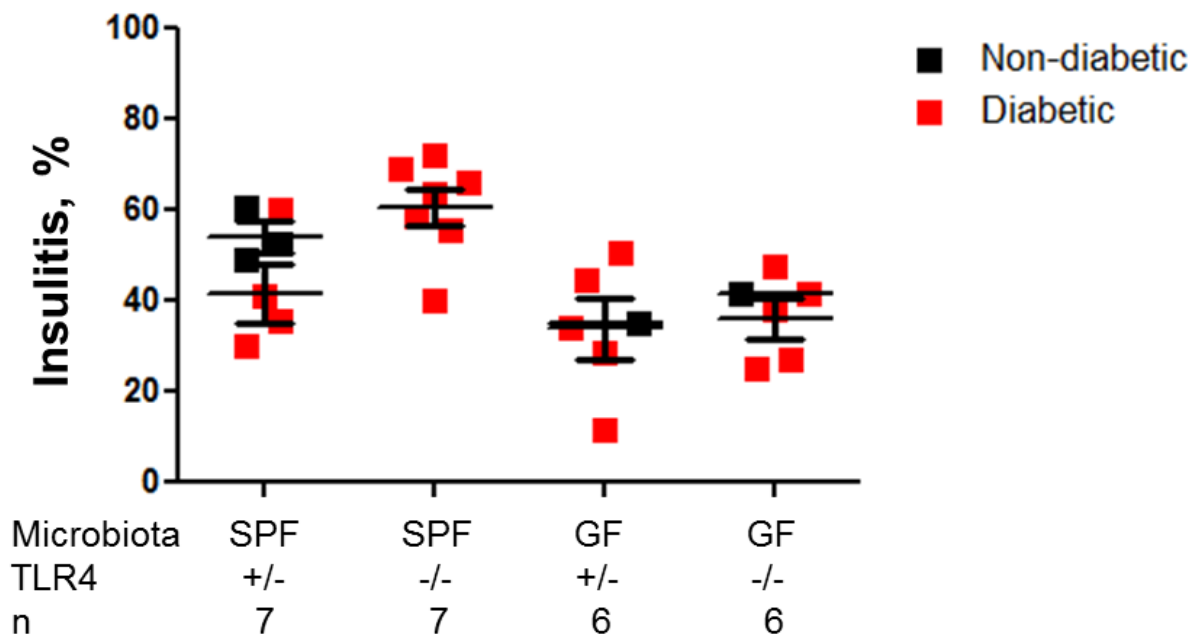
**Figure 20: Insulitis observed in 30 week old TLR4-negative SPF and GF NOD mice**

Bona fide insulitis (percentage of total islets) in diabetic (red) and nondiabetic (black) 30 week old female SPF TLR4-heterozygous, TLR4-negative littermates, and GF TLR4-heterozygous, TLR4-negative littermates. Deletion of TLR4 signaling in in SPF, but not in GF conditions (p=0.787), accelerated disease development compared with their heterozygous littermates (p=0.042). P values for histopathology were determined by Student's t test. n = number of mice per group.

the cell populations that were efficiently replaced (Figure 21). We saw >95% donor replacement of myeloid cell populations and B cells, and approximately 80%replacement of T cell populations with donor bone marrow.  Therefore, if we observed protection from T1D in the recipients injected with MyD88-negative bone marrow, but not the control mice, we could ascribe the phenotype to the hematopoietic compartment.  Furthermore, we could limit studies of conditional MyD88 deletion to those populations that were efficiently replaced in the protected NOD mice.  We observed the incidence of T1D in the bone marrow chimeras over 30 weeks (Figure 22). The bone marrow chimeras that had MyD88-negative donors were partially, but significantly, protected from T1D compared with those that the received control bone marrow (Figure 23).  However, the protection was incomplete which could be interpreted as: part of the MyD88 phenotype resides in the stromal compartment or part of the phenotype resides in the incompletely replaced hematopoietic cell populations. Thus we had observed significant protection by incomplete deletion of MyD88 in hematopoietic compartment. This did not provide clues as to whether one or more cell populations contributed to the MyD88-negative NOD phenotype. Thus we took a Cre/Lox approach to identify individual cell populations where the absence of MyD88 would generate protection from T1D.
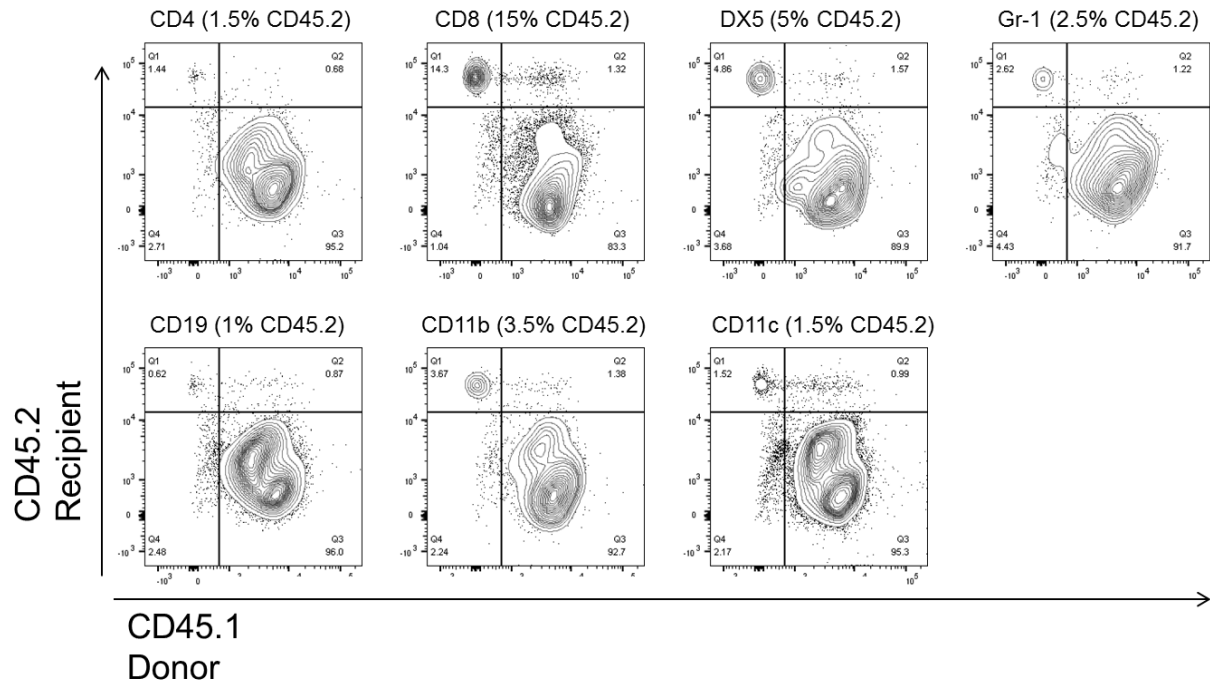
**Figure 21: Hematopoietic cell reconstitution in CD45.1/CD45.2 MyD88–negative bone marrow chimeras**

Reconstitution of the hematopoietic cellular compartment following lethal irradiation and I.V. injection of MyD88-negative bone marrow into female CD45.2 NOD mice. Some, but not all, compartments were efficiently reconstituted with donor bone marrow. In particular peripheral CD8+ T cells were approximately 15% recipient derived.
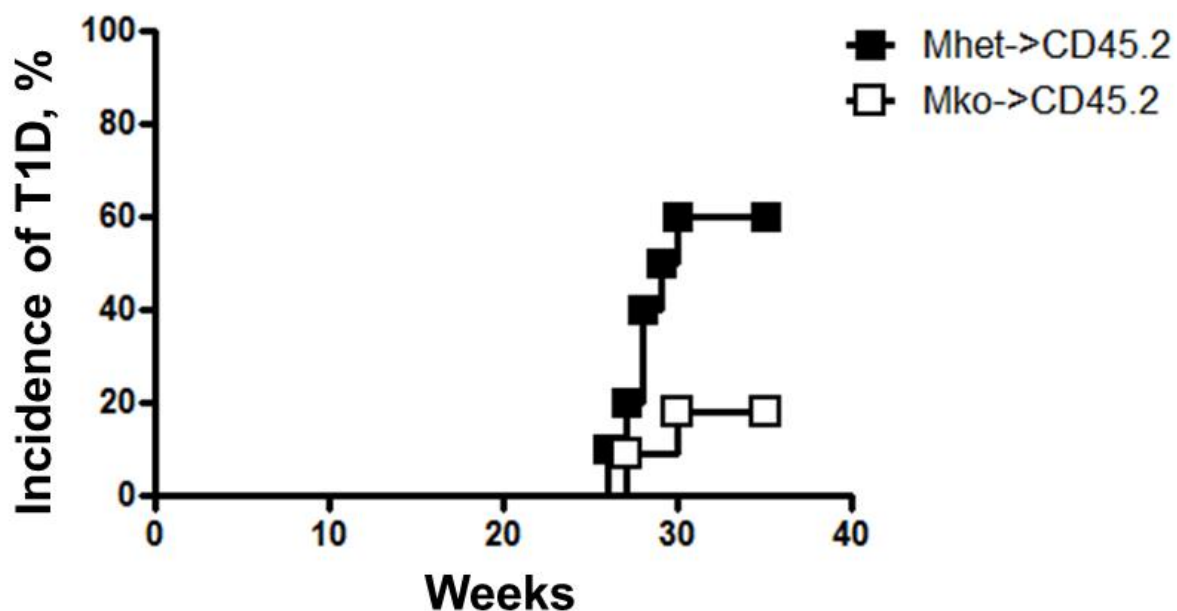
**Figure 22: T1D incidence of CD45.1/CD45.2 MyD88 –negative bone marrow chimera**

Diabetes incidence in CD45.2 female NOD mice lethally irradiated and reconstituted with bone marrow from either MyD88-heterozygous (n=10) or MyD88-negative (n=11) littermate donors. NOD mice reconstituted with MyD88-negative bone marrow exhibited reduced incidence of compared with their heterozygous littermates (P=0.0465). P value was determined using Kaplan-Meier statistics.  n, number of animals per group.

To test the requirement of MyD88 expression in individual cell populations to promote disease, we crossed NOD mice with cell specific promoters driving Cre recombinase to MyD88floxed NODs. We generated homozygous MyD88flox/flox Cre positive NODs and crossed them to NOD MyD88 flox/flox Cre-negative mice to yield Cre positive and Cre negative littermates for observation over 30 weeks. The cells in closest contact with the microbiota are the gut epithelial cells. Deletion of MyD88 was executed by Cre driven by the villin promoter. Observation of T1D incidence in these mice demonstrated no protection in mice when MyD88 was deleted in villin expressing epithelial cells (Figure 24). As expected, histologic examination of the islets showed no change in insulitis between the two cohorts (Figure 25). Next we tested the role of dendritic cells (DC) in T1D, as DCs are central mediators of innate immune responses and in close contact with microbes in the gut and gut associated lymphoid tissue. MyD88 was deleted by expression of Cre recombinase under the control of the CD11c promoter. Observation of T1D incidence in these mice demonstrated no protection in mice when MyD88 was deleted in CD11c expressing DC (Figure 26). In agreement with this finding, histologic examination of the islets showed no change in insulitis between the two cohorts (Figure 27). Although the data is preliminary, deletion of MyD88 in T cells was evaluated using CD4 Cre (Figure 28). Histologic analysis at this stage provided no evidence of protection in NOD mice lacking MyD88 in T cell populations (Figure 29). This data agrees with previous findings that transfer of T cells from MyD88-negative mice to NOD SCID was able to efficiently induce T1D (Wen et al., 2008). Further studies utilizing Lys2 Cre and CD19 Cre for deletion of MyD88 in macrophages and B cells respectively
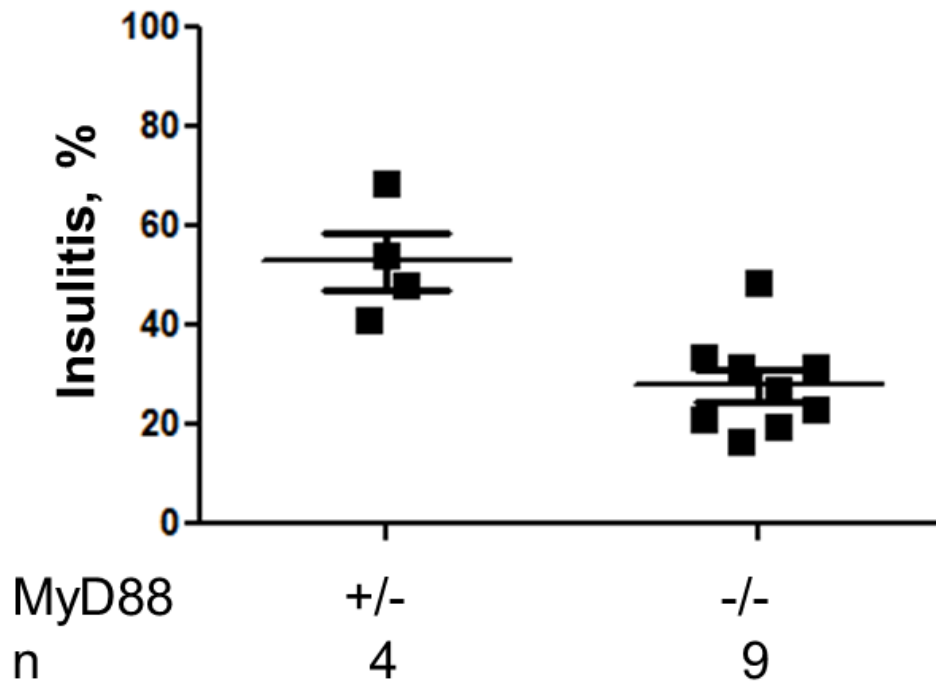
**Figure 23: Insulitis observed in 30 week old CD45.1/CD45.2 MyD88 –negative bone marrow chimera**

Bona fide insulitis (percentage of total islets) in nondiabetic 30 week old female CD45.2 female NOD mice lethally irradiated and reconstituted with bone marrow from either MyD88-heterozygous or MyD88-negative littermate donors. NOD mice reconstituted with MyD88-negative bone marrow exhibited reduced insulitis of compared with their heterozygous littermates (P=0.002). P values for histopathology were determined by Student's t test. n = number of mice per group.

**Figure 24: Deletion of MyD88 in Villin-Cre expressing gut epithelial cells failed to protect from T1D**

Diabetes incidence in SPF NOD female (n=28), NOD MyD88 -negative (n=15), MyD88 flox/flox villin-Cre-negative (n=14) and MyD88 flox/flox villin-Cre-positive (n=17) littermates. Deletion of MyD88 in villin-Cre expressing gut epithelial cells failed to protect from diabetes (P=0.72). P value was determined using Kaplan-Meier statistics. n, number of animals per group.

**Figure 25: Deletion of MyD88 in Villin-Cre expressing gut epithelial cells failed to reduce insulitis**

Bona fide insulitis (percentage of total islets) nondiabetic 30 week old female SPF MyD88 flox/flox villin-Cre− and MyD88 flox/flox villin-Cre-positive littermates. Deletion of MyD88 in villin-Cre expressing gut epithelial cells failed to reduce insulitis (P=0.7264). P value for histopathology were determined by Student's t test. n = number of mice per group.
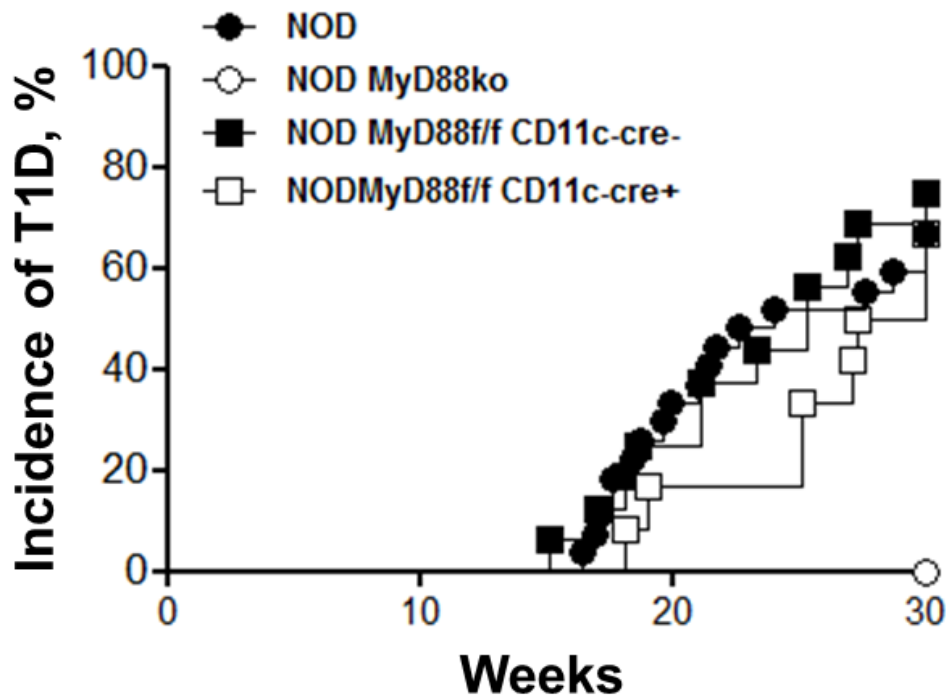
**Figure 26: Deletion of MyD88 in CD11c-Cre expressing dendritic cells failed to protect from T1D**

Diabetes incidence in SPF NOD female (n=28), NOD MyD88 -negative (n=15), and MyD88 flox/flox CD11c-Cre-negative (n=16), MyD88 flox/flox CD11c-Cre-positive (n=12) littermates. Deletion of MyD88 in CD11c expressing dendritic cells failed to protect from diabetes (P=0.848). P value was determined using Kaplan-Meier statistics. n, number of animals per group.

are ongoing.  In summary we have observed that deletion of MyD88 in gut epithelia, DCs, and T cells did not exhibit a reduction in T1D incidence.

We initially did not have access to a B cell specific promoter of Cre on the NOD background.  During the process of backcrossing the C57BL/6 CD19 Cre to the NOD background we designed an alternative approach to test the role of B cell MyD88 signaling.  We utilized a mixed bone marrow chimera approach wherein we injected IgH6-negative NOD with a 1:1 mix of IgH6-negative bone marrow with either MyD88-heterozygous or MyD88-negative bone marrow. IgH6-negative NOD mice lack B cells, therefore donor/recipient bone marrow reconstitution efficiency was not an issue.  This approach yielded NOD mice where all of the B cells present originated from either MyD88 sufficient or deficient donors.  All other hematopoietic cell populations were a 1:1 mix of MyD88 sufficient or deficient cells and MyD88 sufficient IgH6-negative. (Figure 30). We hypothesized that the bone marrow chimeras that received MyD88 deficient B cell would be protected from T1D. We observed the IgH6 mixed bone marrow chimera NOD mice for 30 weeks (Figure 31). Additionally we collected pancreas tissue for histologic evaluation at 30 weeks of age (Figure 32). Interestingly,
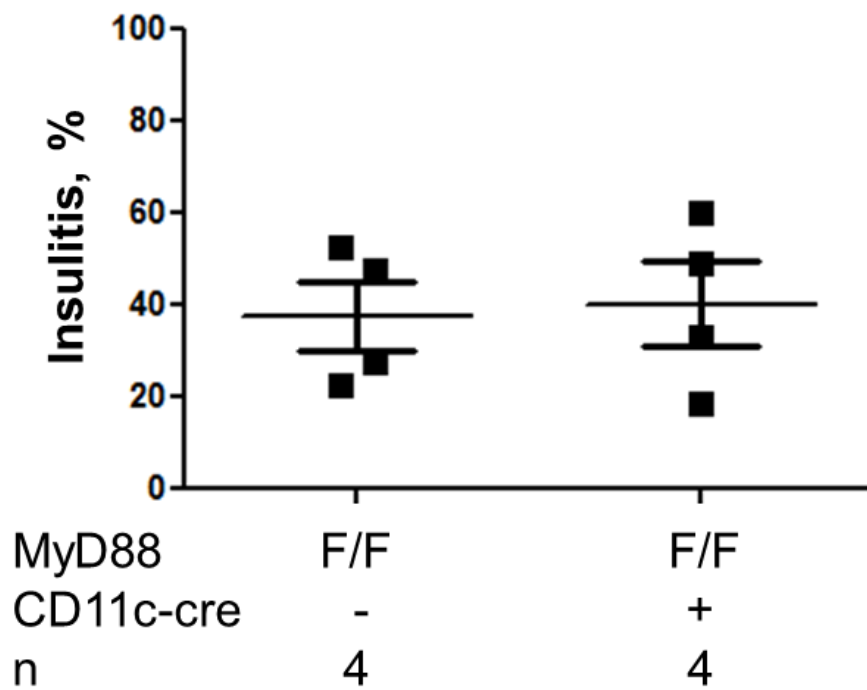
**Figure 27: Deletion of MyD88 in CD11c-Cre expressing dendritic cells failed to reduce insulitis**

Bona fide insulitis (percentage of total islets) nondiabetic 30 week old female SPF MyD88 flox/flox CD11c-Cre-negative and MyD88 flox/flox CD11c-Cre-positive littermates. Deletion of MyD88 in CD11c-Cre expressing dendritic cells failed to reduce insulitis (P=0.8264). P value for histopathology were determined by Student's t test. n = number of mice per group.
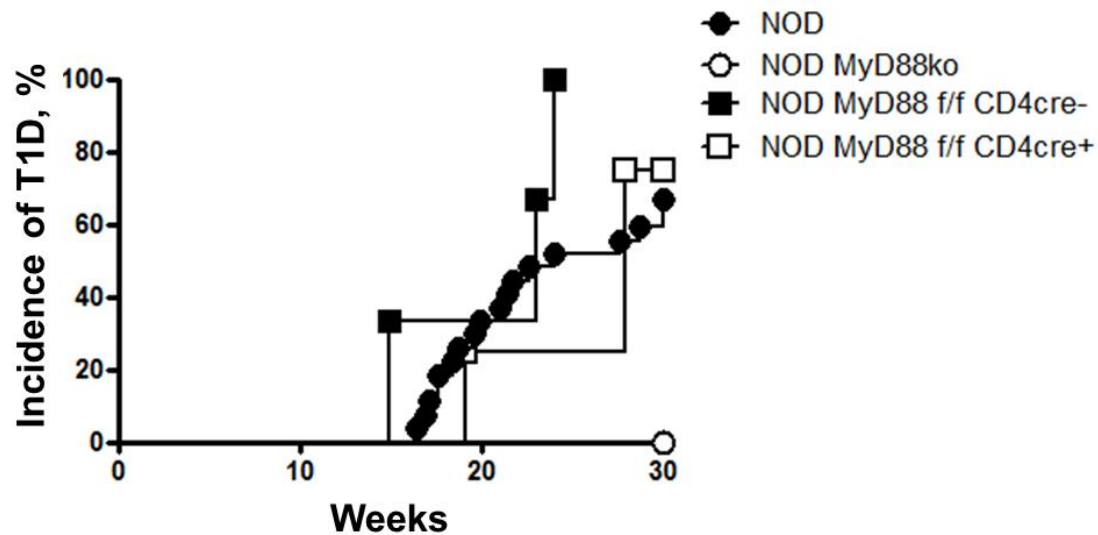
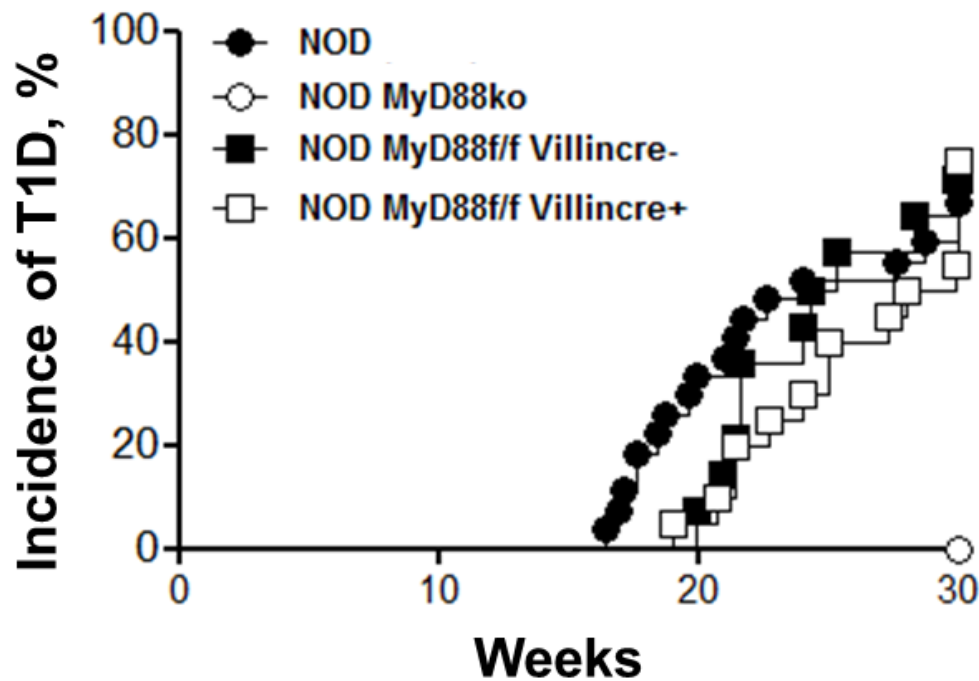**Figure 28: Deletion of MyD88 in CD4-Cre expressing T cells failed to protect from T1D**

Diabetes incidence in SPF NOD female (n=28), NOD MyD88 -negative (n=15), and MyD88 flox/flox CD4-Cre-negative (n=3), MyD88 flox/flox CD4-Cre-positive (n=4) littermates. Deletion of MyD88 in CD4 expressing T cells failed to protect from diabetes (P=0.574). P value was determined using Kaplan-Meier statistics. n, number of animals per group.

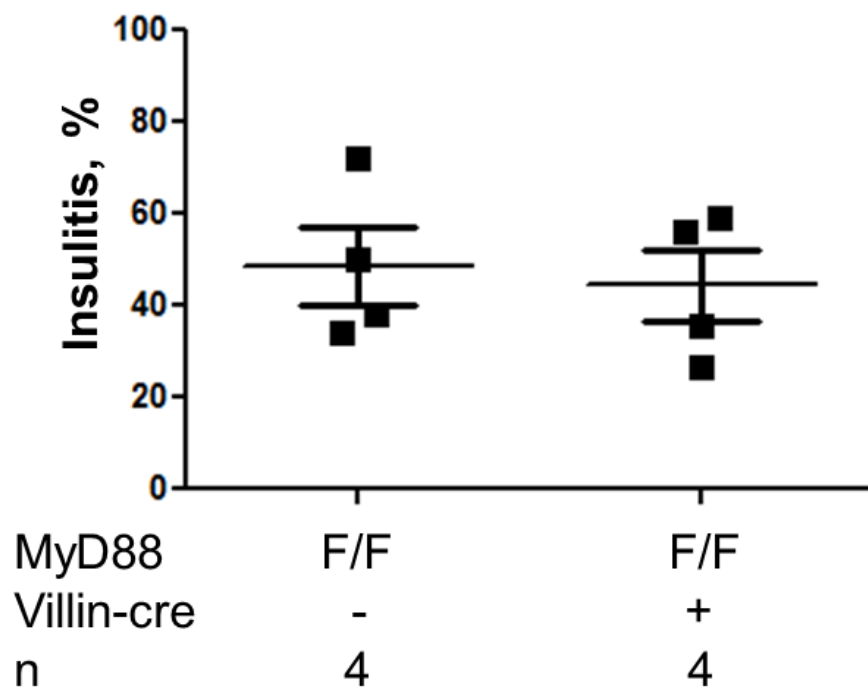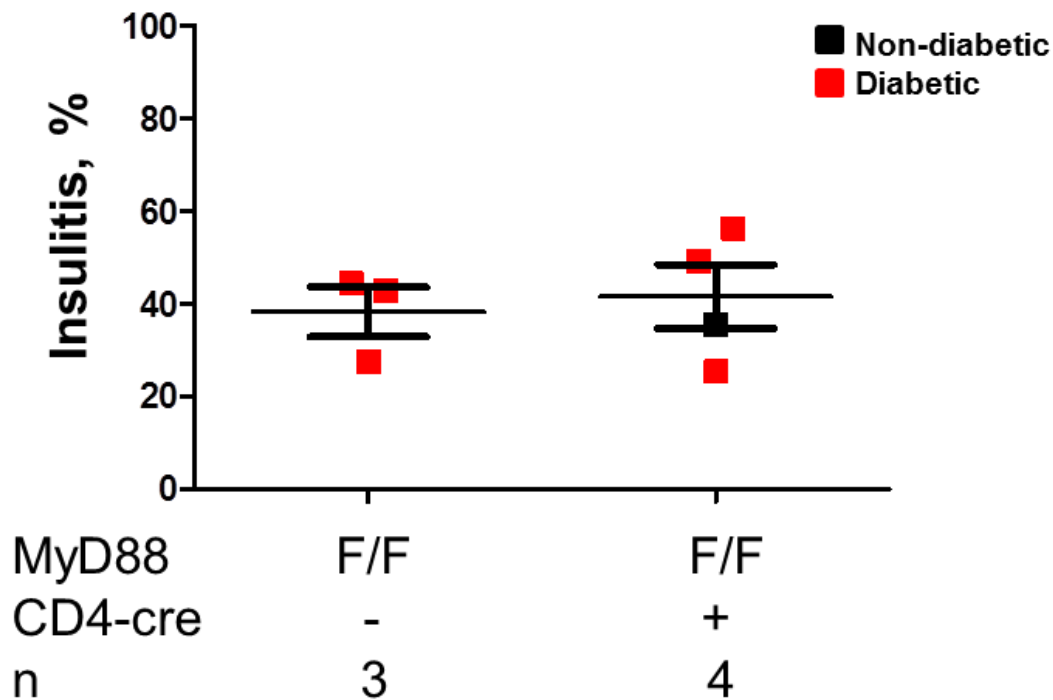**Figure 29: Deletion of MyD88 in CD4-Cre expressing T cells failed to eliminate insulitis**

Bona fide insulitis (percentage of total islets) in diabetic (red) and nondiabetic (black) 30 week old female SPF MyD88 flox/flox CD4-Cre-negative and MyD88 flox/flox CD4-Cre-positive littermates. Deletion of MyD88 in CD4-Cre expressing T cells failed to prevent insulitis. n = number of mice per group.
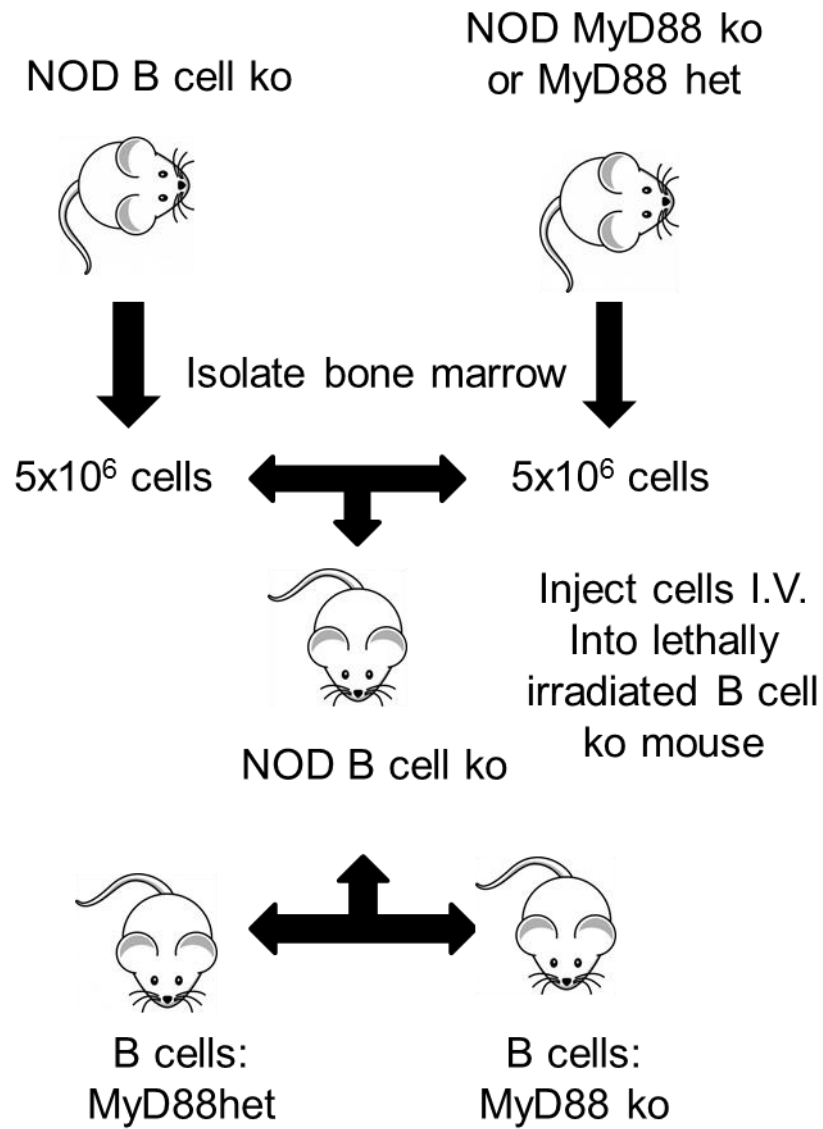
**Figure 30: Experimental design for IgH6-negative bone marrow chimera**
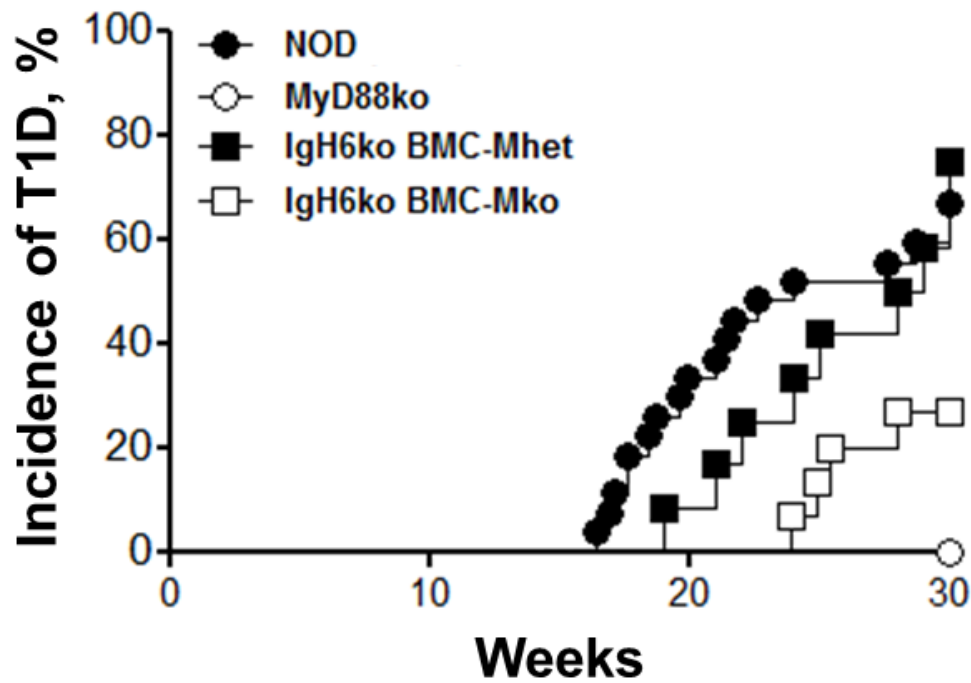
**Figure 31: B cell knockout mixed bone marrow chimera**

Diabetes incidence in SPF NOD female (n=28), NOD MyD88 -negative (n=15), and IgH6-negative littermates receiving either MyD88-heterozygous (n=12) or MyD88-heterozygous (n=15) donor bone marrow. NOD mice reconstituted with B cells that lacked MyD88 had reduced T1D incidence compared with those whose B cells were derived from MyD88-heterozygous precursors (P=0.0158). P value was determined using Kaplan-Meier statistics.  n, number of animals per group.
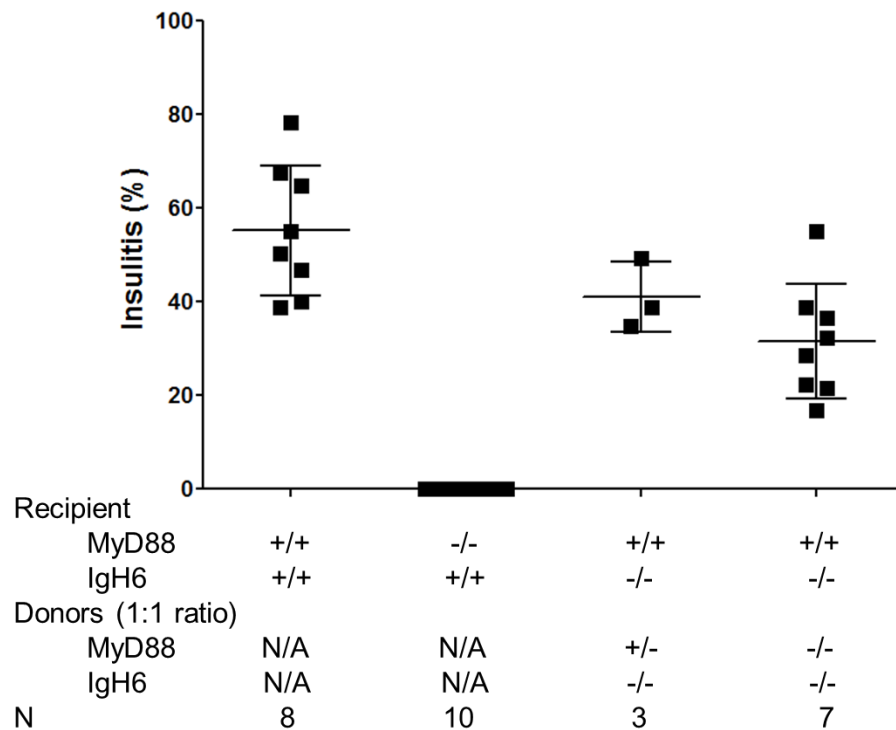
**Figure 32: Insulitis observed in 30 week old B cell mixed bone marrow chimeras**

Bona fide insulitis (percentage of total islets) nondiabetic 30 week old female SPF NOD female (n=28), NOD MyD88 -negative (n=15), and IgH6-negative littermates receiving either MyD88-heterozygous (n=12) or MyD88-heterozygous (n=15) donor bone marrow. NOD mice reconstituted with B cells that lacked MyD88 had reduced insulitis compared with wild-type NOD levels (p<0.01). However, there was no reduction when compared with mice whose B cells were derived from MyD88-heterozygous precursors (P>0.05). P values for histopathology were determined by ANOVA and Bonferroni's Multiple Comparison Test.

the NOD mice lacking MyD88 in their B cells demonstrated significant protection from T1D compared with the control mice that were reconstituted with MyD88 sufficient B cells.

A major caveat of this experimental design is that if the phenotype is dominant in the mixed hematopoietic cell populations, you cannot attribute the effect to the B cells specifically. For example, the presence of 50% of the macrophages lacking MyD88 might create the protection observed. To address this issue, we performed another mixed bone marrow chimera, injecting a 1:1 ratio of NOD and NOD MyD88-negative or MyD88-heterozygous bone marrow into a lethally irradiated (1200 rads) NOD recipient. If the MyD88 phenotype was dominant, then the mix of NOD and NOD MyD88-negative bone marrow would induce protection from T1D development. If so, we would conclude that the IgH6 bone marrow chimera result could not be specific to deletion of MyD88 in B cells. If the MyD88 phenotype is not dominant, we would expect to see similar development of T1D in the NOD mice that received NOD bone marrow mixed with MyD88-negative or MyD88-heterozygous bone marrow. We observed the chimeras for T1D incidence (Figure 33) and collected pancreas for histologic evaluation at 30 weeks (Figure 34). We saw no reduction in T1D incidence, which we interpreted as a lack of dominance of the MyD88 phenotype in the IgH6 bone marrow chimera. Therefore, we conclude that MyD88 signaling in B cells promotes T1D.

Deletion of MyD88 in B cells does not completely restore the100% protection observed in the global NOD MyD88 phenotype. It is likely that the microbiota-dependent MyD88 activation in another cell population contributes to disease development. At backcross N8 we established observation groups for CD19 Cre driven deletion of

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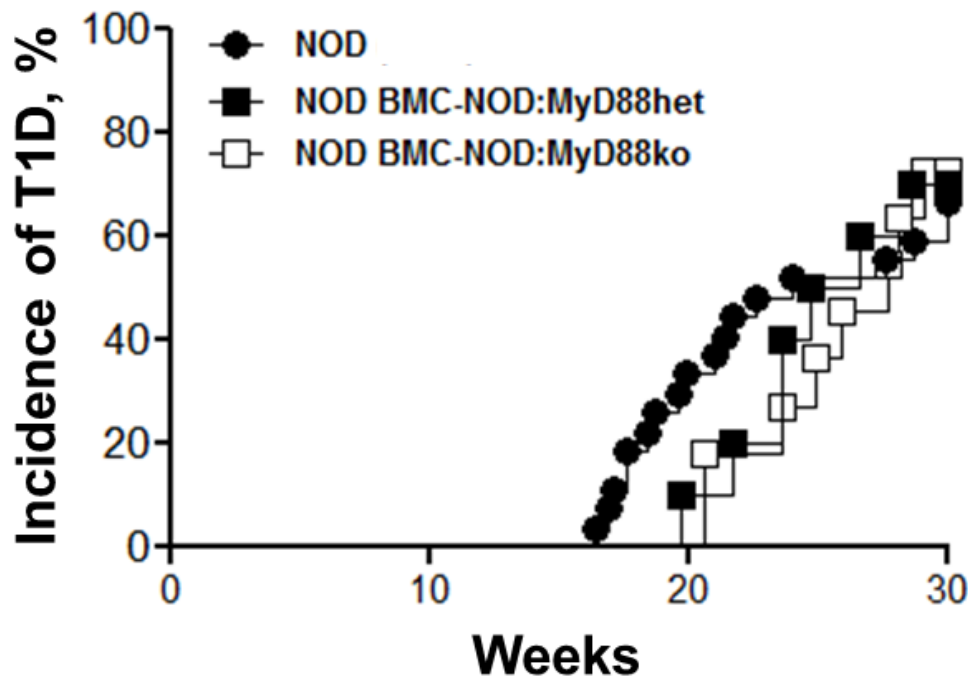
**Figure 33: MyD88 phenotype dominance in a mixed bone marrow chimera**

Diabetes incidence in SPF NOD female (n=28) and NOD littermates receiving a 1:1 mix of NOD bone marrow and either MyD88-heterozygous (n=10) or MyD88-heterozygous (n=11) donor bone marrow. NOD mice reconstituted with NOD:MyD88-negative bone marrow did not exhibit a reduction in T1D incidence when compared with those that received NOD:MyD88-heterozygous bone marrow (P=0.91). P value was determined using Kaplan-Meier statistics. n, number of animals per group.
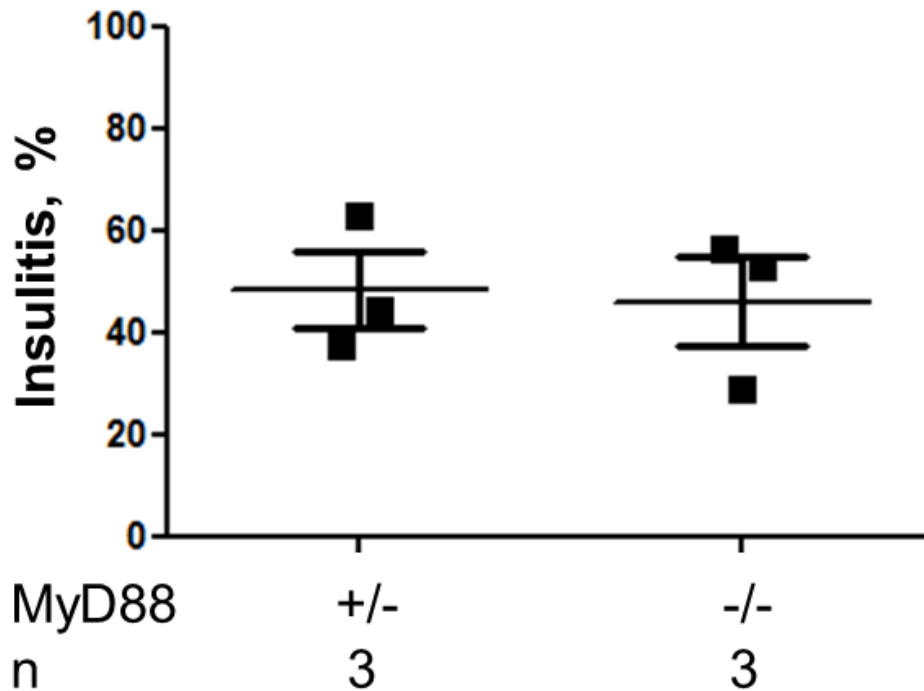
**Figure 34: Insulitis observed in 30 week old mixed bone marrow chimeras to test for dominance of the MyD88 phenotype.**

Bona fide insulitis (percentage of total islets) nondiabetic 30 week old female SPF NOD littermates receiving a 1:1 mix of NOD bone marrow and either MyD88-heterozygous or MyD88-heterozygous donor bone marrow. NOD mice reconstituted with NOD:MyD88-negative bone marrow did not exhibit a reduction in insulitis when compared with those that received NOD:MyD88-heterozygous bone marrow (P=0.8498). P value for histopathology were determined by Student's t test. n = number of mice per group.

MyD88. Preliminary results from these mice showed protection from T1D in the mice with MyD88 deleted in B cells (Figure 35). However, the incidence in the control B cell MyD88 sufficient mice did not reach the level of T1D incidence we have typically observed in our colony. Further backcrosses are being done and further observation groups will be established and tested for T1D. Although preliminary, this data supports the finding that MyD88 signaling in B cells is a microbiota dependent promoter of disease development.

Previous studies had shown that NOD mice deficient in B cells (IgH6-negative) are strongly protected from development of T1D. Additionally, NOD IgH6-negative mice whose B cells were rescued by expression of a transgenic BCR recognizing hen egg lysozyme (HEL) are also protected from T1D. These studies were done by observing mice in SPF conditions. MyD88 is a critical regulator of cell development and activation in B cells. One possibility was that the requirement for MyD88 in B cells we observed was fundamentally a requirement for B cell development in the NOD mouse model of diabetes. We hypothesized that NOD IgH6-negative and NOD IgH6-negative HEL transgene positive (HELtg-positive) mice rederived into GF conditions would result in rescue of the disease phenotype. It was important to demonstrate that GF IgH6-negative HELtg-positive had restored B cell development and function, as was observed in SPF conditions. We evaluated serum IgM concentrations by ELISA (Figure 36) and restoration of lymphoid architecture by histologic analysis in the spleen and peripheral lymph nodes (Figure 37). We were able to replicate the reported phenotype of the IgH6-negative mice and IgH6-negative HELtg-positive, demonstrating complete protection at 30 weeks in SPF conditions by disease incidence (Figure 38) and histologic scoring
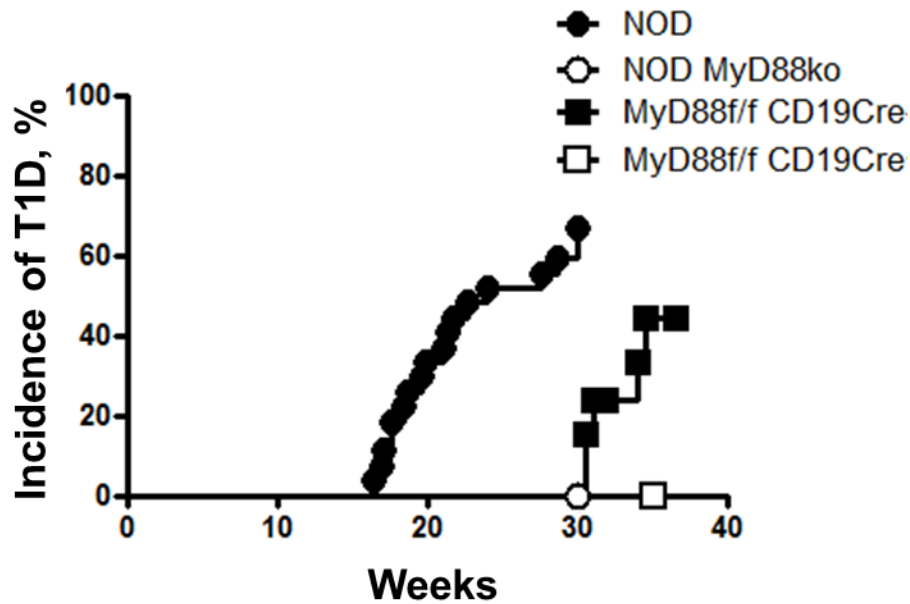
**Figure 35- Deletion of MyD88 in CD19-Cre expressing B cells**

Diabetes incidence in SPF NOD female (n=28), NOD MyD88 -negative (n=15), and

MyD88 flox/flox CD19-Cre-negative (N8) (n=13), MyD88 flox/flox CD19-Cre-positive

(N8) (n=11) littermates. Deletion of MyD88 in CD19 expressing B cells reduced the

incidence of T1D (P=0.0148). However, the incidence in the CD19-Cre-negative

littermates was reduced compared to wild type NOD, which suggested additional

backcrosses were necessary.  P value was determined using Kaplan-Meier statistics.

n, number of animals per group. N, number of backcrosses to the NOD background
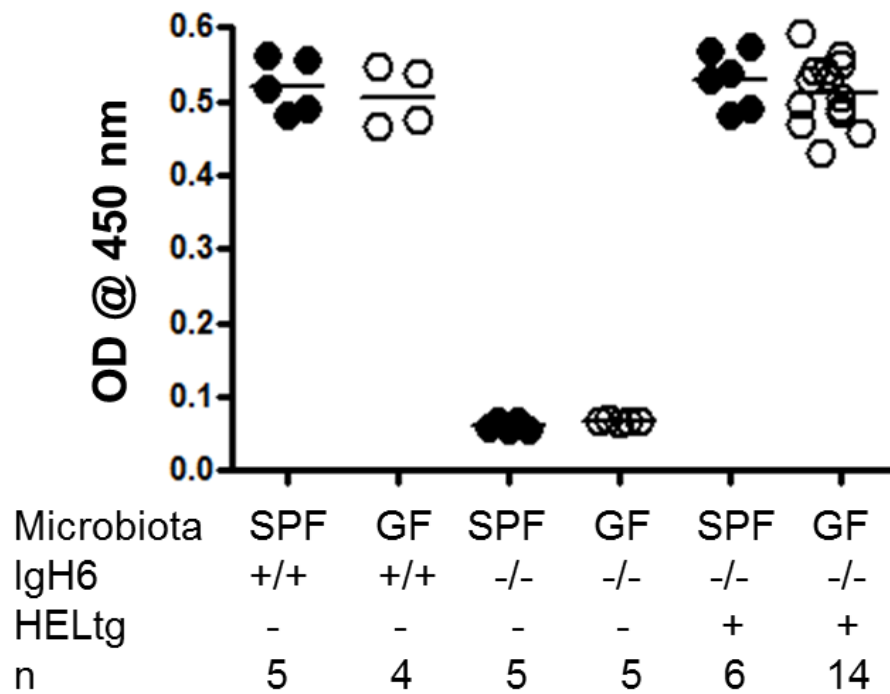
**Figure 36: Restoration of serum antibody production in IgH6-negative HELtg NOD mice**

Serum IgM concentrations were evaluated by ELISA. No serum IgM was detected in SPF and GF IgH6-negative mice. No difference was observed in serum IgM concentrations for SPF and GF NOD mice (P>0.05), SPF and GF IgH6-negative HELtg+ NOD mice (P>0.05), or between SPF and GF NOD and IgH6-negative HELtg+ NOD mice (P>0.05). P values for antibody concentrations were determined by ANOVA and Bonferroni's Multiple Comparison Test. n, number of animals per group.
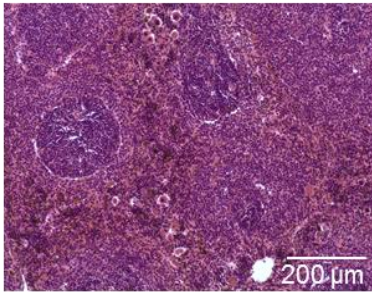
**Figure 37: Restoration of splenic architecture in IgH6-negative HELtg+**

Spleens from IgH6-heterozygous, IgH6-negative, and IgH6-negative HELtg+ were

sectioned and stained with H&E to visualize lymphoid architecture. Representative

images from each genotype are shown.
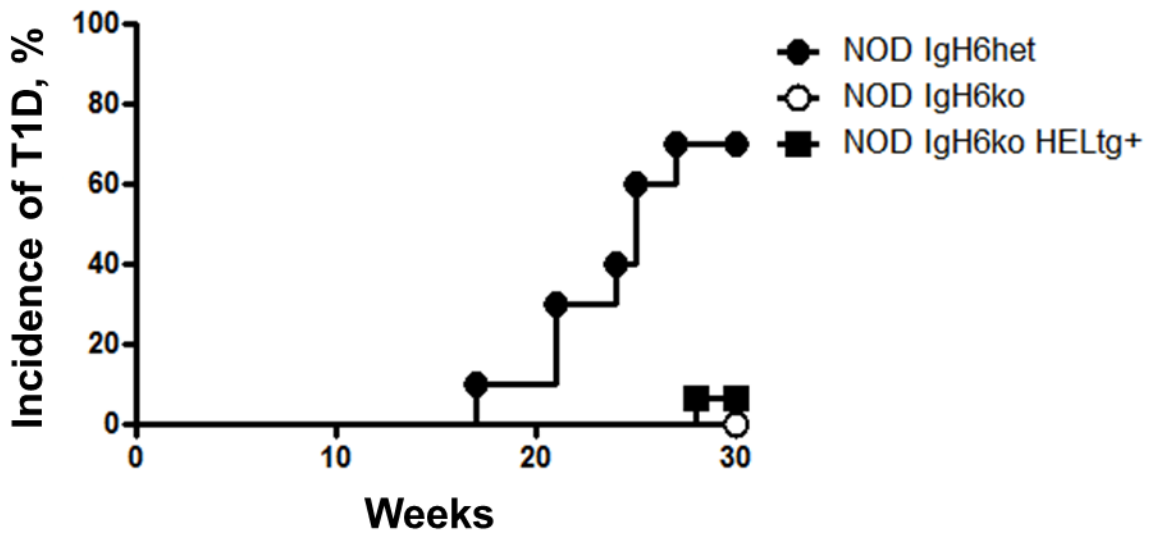
**Figure 38: Incidence of T1D in SPF B cell knockout and HEL-BCR transgene expressing NOD mice**

Diabetes incidence in SPF NOD IgH6-heterozygous female (n=10), NOD IgH6-negative (n=18), and NOD IgH6-negative HELtg+ (n=15). Deletion of B cells (P=0.0001) and rescue of B cells with an HEL-BCR (P=0.0003) in NOD mice protects from T1D. P value was determined using Kaplan-Meier statistics.  n, number of animals per group.

(Figure 39). NOD IgH6-negative NOD mice in GF conditions remained protected from

diabetes (Figure 40).  We also observed very low levels of insulitis in the IgH6-negative

GF NOD mice and IgH6-negative HELtg-positive mice (Figure 41). Thus we concluded

that the presence of polyclonal, and likely islet reactive BCR bearing, B cells was

necessary for disease pathogenesis. This requirement was not dependent on signaling

from the microbiota and represented a separate phenomenon from that of the protection

induced by deletion of MyD88 in NOD B cells.

## Chapter 5: Effectors of microbiota-dependent signaling

A goal of this project was to attempt to identify downstream effectors of the protective and disease promoting pathways that have been described here. One obvious candidate tolerance induction was interleukin 10 (IL-10). IL-10 is a pleotropic cytokine that has well described immunoregulatory activity over a broad range of cells in the immune system.  Best studied are IL-10's anti-inflammatory properties. IL-10 binds to its receptor and allows the dimerization of signal transducer and activator of transcription 3 (STAT3). Activation of STAT3 allows the transcription factor to traffic to the nucleus and alters the expression of a wide variety of genes.  An important regulatory targets of STAT3 include suppressor of cytokine signaling 3 which suppresses inflammatory cytokines including tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), and IL-1 (Williams et al., 2004). We hypothesized that IL-10 could be an important effector utilized by a MyD88-independent, microbiota-dependent protective pathway(s).  First it was important to demonstrate whether or not IL-10 directly influenced T1D development. IL-10-negative NOD mice were observed for incidence of
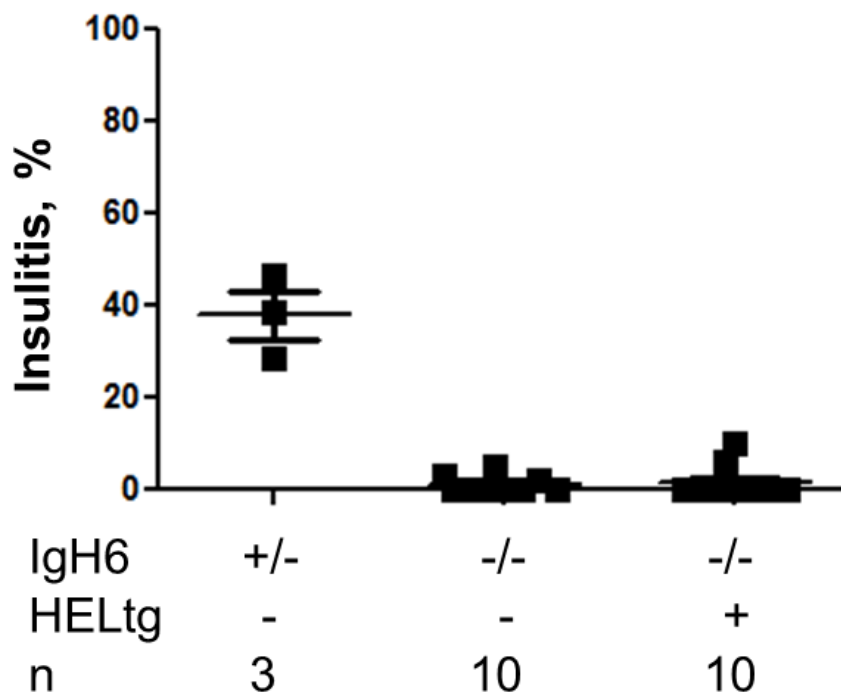
**Figure 39: Insulitis observed in SPF B cell knockout and HEL-BCR transgene expressing NOD mice**

Insulitis in SPF NOD IgH6-heterozygous female, NOD IgH6-negative, and NOD IgH6-negative HELtg+. Deletion of B cells (P<0.0001) and rescue of B cells with an HEL-BCR (P<0.0001) in NOD mice protects from insulitis. P value for histopathology were determined by Student's t test. n = number of mice per group.
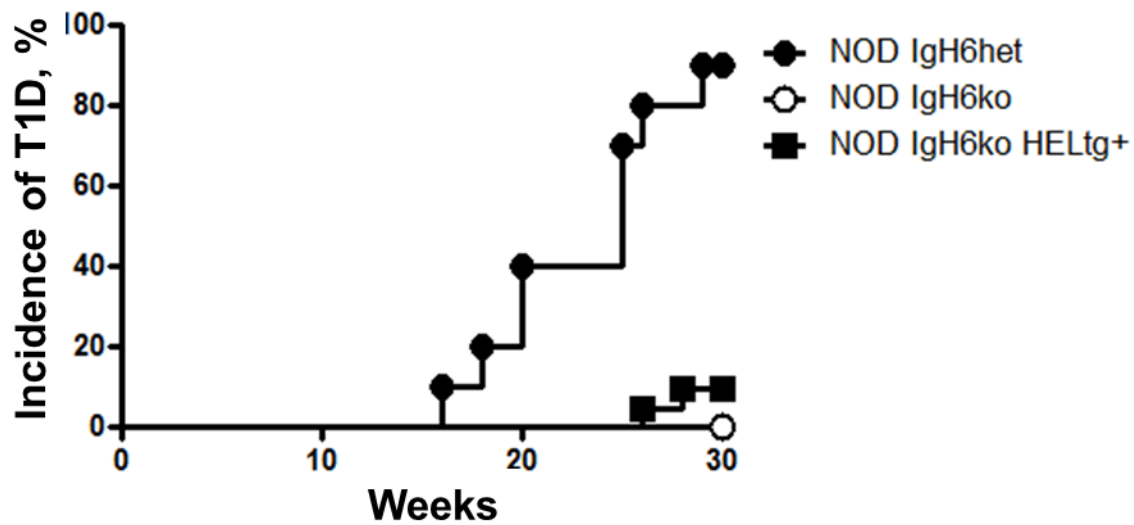
**Figure 40: Incidence of T1D in GF B cell knockout and HEL-BCR transgene expressing NOD mice**

Diabetes incidence in GF NOD IgH6-heterozygous female (n=11), NOD IgH6-negative (n=19), and NOD IgH6-negative HELtg+ (n=21). Deletion of B cells (P<0.0001) and rescue of B cells with an HEL-BCR (P<0.0001) in NOD mice protects from T1D. P value was determined using Kaplan-Meier statistics.  n, number of animals per group.
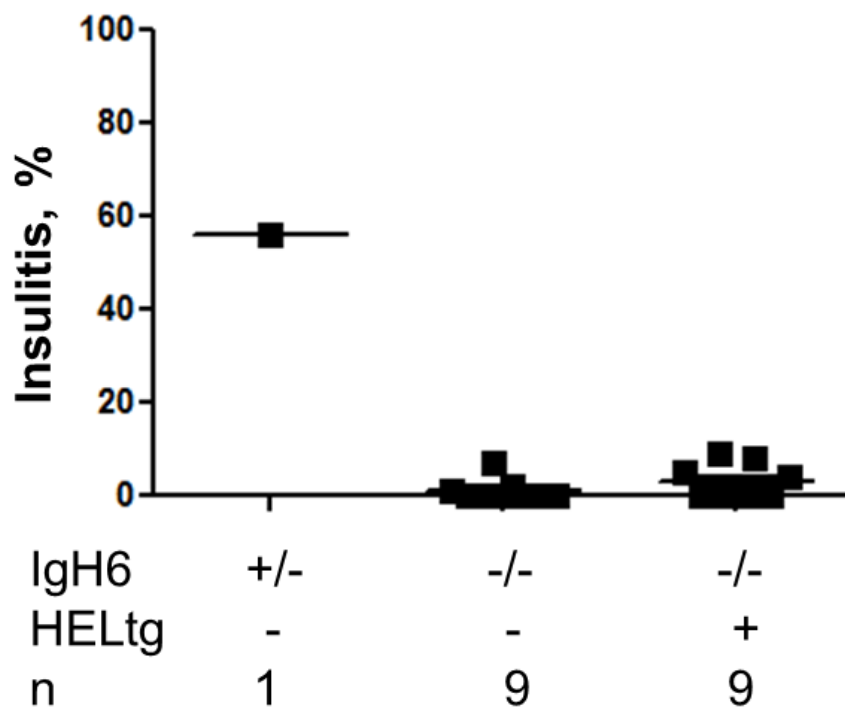
**Figure 41: Insulitis observed in GF B cell knockout and HEL-BCR transgene expressing NOD mice**

Insulitis in GF NOD IgH6-heterozygous female, NOD IgH6-negative, and NOD IgH6-negative HELtg+. Deletion of B cells and rescue of B cells with an HEL-BCR in NOD mice protects from insulitis. n = number of mice per group.

diabetes over 30 weeks (Figure 42). Interestingly, IL-10-negative mice had a lower incidence of disease than their heterozygous littermates. However, this unexpected result must be interpreted in the context of an important caveat. Our mouse facility is not *Helicobacter pylori* free. NOD IL-10-negative mice colonized with *Helicobacter pylori* spontaneously develop rectal prolapse and must be euthanized. Therefore, approximately 30% of the IL-10-negative, but not the IL-10 heterozygous, NOD mice under observation cannot be observed for T1D to 30 weeks. Thus if there was any correlation between development of rectal prolapse and diabetes, the IL-10-negative cohort would be skewed. Only mice that did not develop rectal prolapse would be counted in the diabetes survival analysis. However, no evidence currently exists to link development of rectal prolapse and T1D in NOD IL-10-negative mice. IL-10 may have a direct role in diabetes pathology or our observation may be an artifact of the model system.

Although we saw reduced incidence of T1D in IL-10-negative NOD mice, the interpretation of the results was potentially confounded by the development of rectal prolapse in the IL-10-negative cohort. However, a recent report demonstrated that the development of rectal prolapse was dependent on MyD88 (Hoshi et al., 2012). Therefore by crossing IL-10-negative NOD mice to MyD88-negative NOD mice we could establish IL-10-negative and IL-10-heterozygous littermates on a NOD MyD88-negative background. Both cohorts of mice would not develop rectal prolapse and could be observed for diabetes incidence over 30 weeks (Figure 43). Pancreas tissue was collected at 30 weeks of age and histologic scoring of insulitis was performed (Figure 44). Both diabetes incidence and histology scoring indicated that IL-10 did not provide
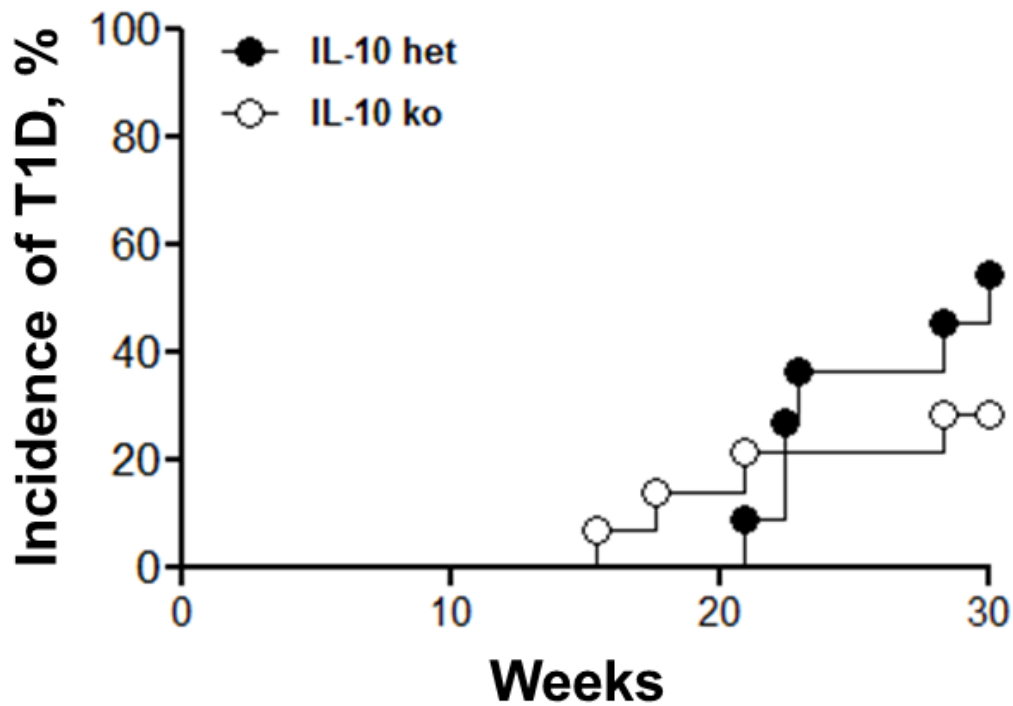
**Figure 42: Ablation of IL-10 signaling did not protect from T1D**

Diabetes incidence in NOD female IL-10-heterozygous (n=11), IL-10-negative (n=14)

littermates. Removal of IL-10 signaling did not reduce incidence of disease compared

with their heterozygous littermates. Although there was a trend toward lower disease

incidence in the IL-10-negative NOD mice it was not statistically significant (P=0.2796).

P value was determined using Kaplan-Meier statistics.  n, number of animals per group.
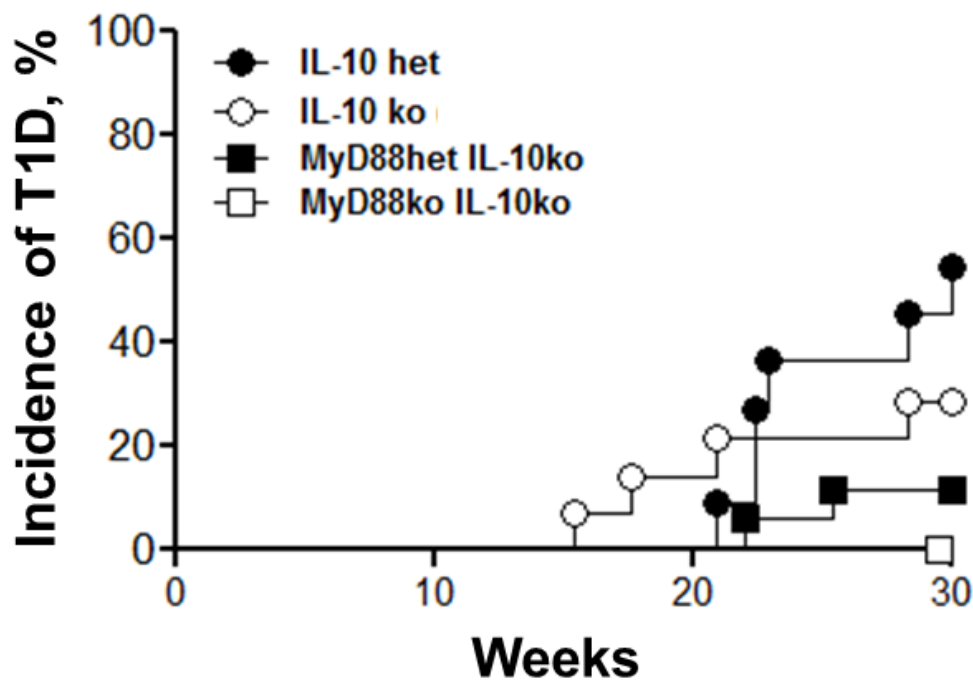
**Figure 43: Ablation of IL-10 signaling in the absence of MyD88 failed to rescue T1D**

Diabetes incidence in NOD female IL-10-heterozygous (n=11), IL-10-negative (n=14) littermates, and IL-10-negative MyD88-heterozygous (n=17), IL-10-negative MyD88-negative (n=32) littermates. Removal of IL-10 signaling in the absence of MyD88 failed to rescue disease development compared with their MyD88-heterozygous littermates (P=0.0488). P value was determined using Kaplan-Meier statistics. n, number of animals per group.

**Figure 44: Insulitis observed in 30 week old MyD88-negative IL-10-negative NOD mice**

Bona fide insulitis (percentage of total islets) in nondiabetic 30 week old female IL-10-heterozygous, IL-10-negative littermates, and MyD88-heterozygous IL-10-/− , MyD88-negative IL-10-negative littermates. Deletion of IL-10 signaling in the absence of MyD88 did not rescue insulitis in double knockout NOD mice compared with their MyD88-heterozygous IL-10-heterozygous littermates ($p>0.05$). P values for histopathology were determined by ANOVA and Bonferroni's Multiple Comparison Test. n = number of mice per group.

microbiota-dependent protection in the absence of MyD88. Thus IL-10 did not appear to be a critical effector of MyD88-independent, microbiota-dependent protective signal(s).

The NADPH oxidase complex is required to generate reactive oxygen species (ROS) in many cell populations. For example, NADPH oxidase is required to generate ROS in macrophage and neutrophil oxidative burst.  However, ROS production has been implicated in many aspects of immune function: bacterial killing (Vazquez-Torres and Fang, 2001), alter T cell function (Hildeman et al., 1999), Islet β cell survival (Weaver et al., 2015). Recently, a dominant negative p47phox mutation of the NADPH oxidase complex mouse was engineered (ROS-negative). ROS-negative NOD mice were generated and observed for diabetes incidence.  Incidence data was published from two separate facilities, in one location T1D incidence was approximately 35% (Tse et al., 2010), in the other it was approximately 5% (Thayer et al., 2011). ROS generation is downstream of MyD88 activation in many cell types. Interestingly, the incidence of T1D in ROS-negative NOD mice varied between facilities.  We hypothesized that ROS could be a downstream effector of MyD88 signaling which promoted diabetes.  To test this hypothesis we rederived NOD ROS-negative mice into GF housing conditions. We observed ROS-negative NOD incidence in SPF conditions at the University of Chicago animal facilities (Figure 45). Histologic examination of the islets showed reduced insulitis in the ROS-negative compared with its ROS-heterozygous littermates (Figure 46). Thus we demonstrated T1D incidence similar to that of one of the previous facilities in our SPF housing (approximately 35%). Next we provided the ROS NOD mice with Sulfamethoxazole/Trimethoprim antibiotic (Bactrim*) ad libitum* in their drinking water. We had previously observed that this treatment protocol increased T1D incidence in

**Figure 45: T1D incidence of SPF ROS-negative NOD mice**

Diabetes incidence in NOD female ROS-heterozygous (n=31), ROS-negative (n=16)

littermates. Ablation of ROS signalings reduced incidence of disease compared with

their heterozygous littermates (P=0.0145). P value was determined using Kaplan-Meier

statistics. n, number of animals per group.

**Figure 46: Insulitis observed in 30 week old SPF ROS-negative NOD mice**

Bona fide insulitis (percentage of total islets) in nondiabetic 30 week old female ROS-
heterozygous, ROS-negative littermates. Deletion of ROS signaling rescued insulitis

compared with their ROS-heterozygous littermates (P=0.008). P values for

histopathology were determined by Student's t test. n = number of mice per group.

MyD88-negative NOD mice. We hypothesized that addition of antibiotic to the drinking water would similarly enhance the incidence of diabetes. We observed ROS-negative and ROS-heterozygous NOD littermates for T1D incidence in SPF conditions with Bactrim drinking water (Figure 47). Histologic examination of the islets showed no reduction in insulitis in the ROS-negative compared with its ROS-heterozygous littermates (Figure 48). Interestingly there was a slight decrease, although not statistically significant, in the incidence of diabetes comparing ROS-negative SPF and ROS-negative SPF with Bactrim. And no significant change was seen in the histologic scoring.  In the true test of microbiota-dependent protection GF ROS-negative and ROS-heterozygous littermates were observed until 30 weeks of age for T1D incidence (Figure 49). Histologic examination of the islets showed significantly reduced insulitis in the GF ROS-negative compared with its GF ROS-heterozygous littermates (Figure 50). Surprisingly, The GF ROS-negative incidence (<10%) was also significantly lower than what we observed in SPF conditions (35%). The GF incidence data actually recapitulated the incidence data from the initial incidence studies that demonstrated extremely lower incidence in one SPF colony (Thayer et al., 2011). This raised the interesting idea that there is a component of the microbiota capable of increasing T1D incidence in ROS-negative NOD mice. One candidate microbe is *Lactobacillus murinus*, a potent inducer of ROS.  A recent study has linked MyD88 induced ROS generation to efficient control of *L. murinus* (Ichikawa et al., 2012).  Therefore, we colonized GF ROS-negative NOD mice with monocultured *L. murinus* to evaluate a single, strong ROS inducing, microbe's ability to rescue T1D (Figure 51). Histologic examination of the islets showed significantly enhanced insulitis in the GF ROS-negative compared with its
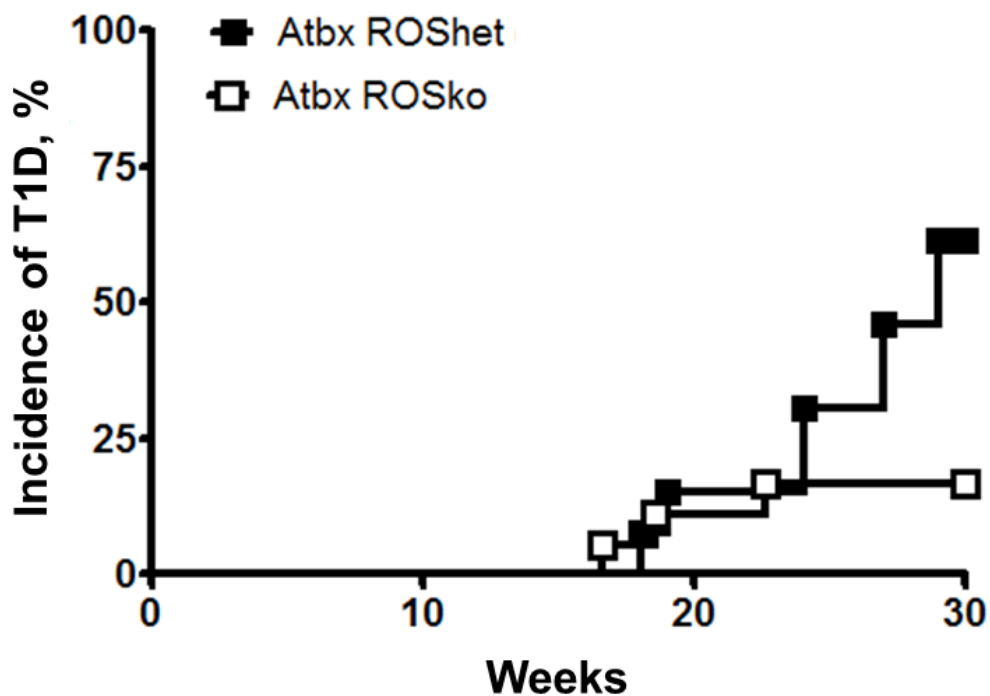
**Figure 47: T1D incidence of SPF antibiotic treated ROS-negative NOD mice**

Diabetes incidence in SPF NOD female ROS-heterozygous (n=31) and ROS-negative (n=16) littermates treated with antibiotics in their drinking water. Ablation of ROS signaling reduced incidence of disease compared with their heterozygous littermates (P=0.0217). P value was determined using Kaplan-Meier statistics.  n, number of animals per group.
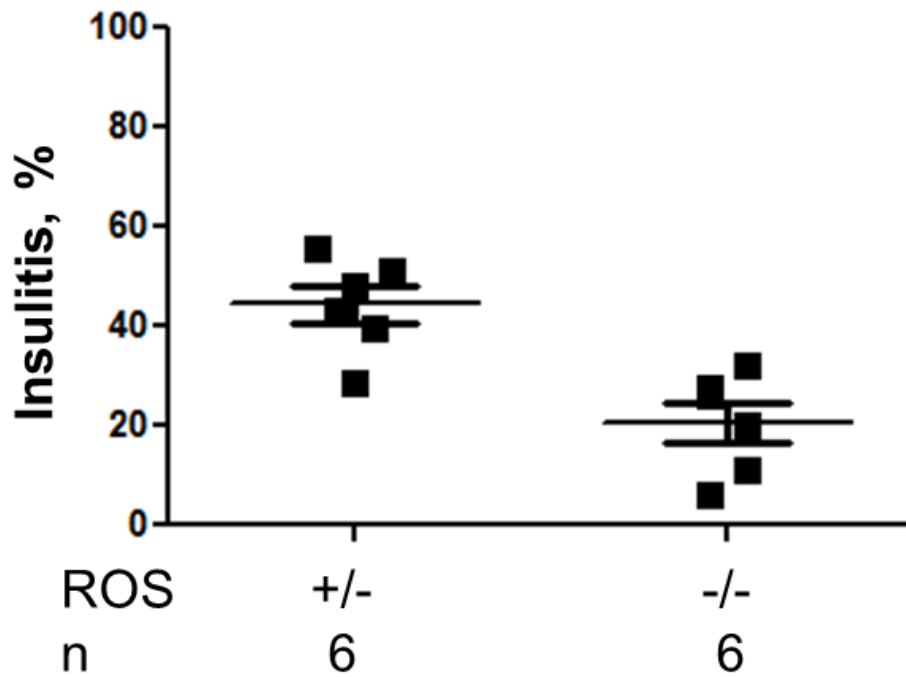
**Figure 48: Insulitis observed in 30 week old SPF antibiotic treated ROS-negative NOD mice**

Bona fide insulitis (percentage of total islets) in nondiabetic 30 week old female ROS-heterozygous and ROS-negative littermates treated with antibiotics in their drinking water. Deletion of ROS signaling rescued insulitis compared with their ROS-heterozygous littermates (P=0.0019). P values for histopathology were determined by Student's t test. n = number of mice per group.
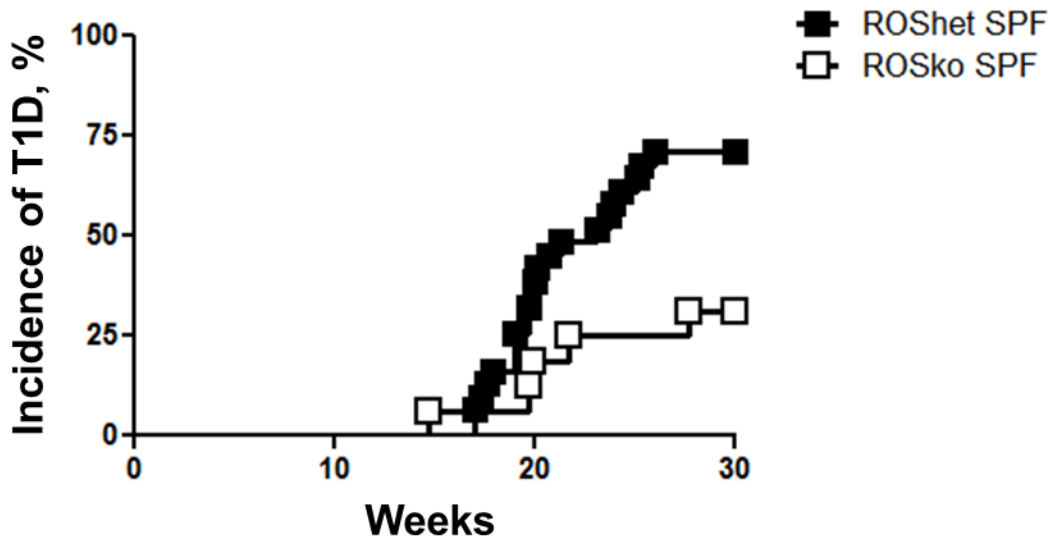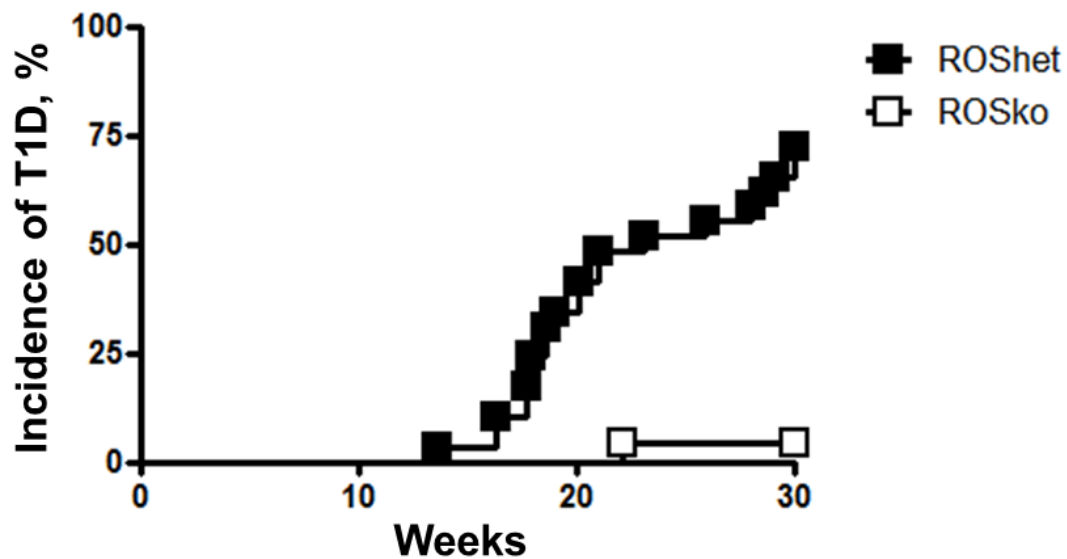
**Figure 49: T1D incidence of GF ROS-negative NOD mice**

Diabetes incidence in GF NOD female ROS-heterozygous (n=29), ROS-negative (n=24) littermates. Ablation of ROS signaling reduced incidence of disease compared with their heterozygous littermates (P=0.001). P value was determined using Kaplan-Meier statistics.  n, number of animals per group.

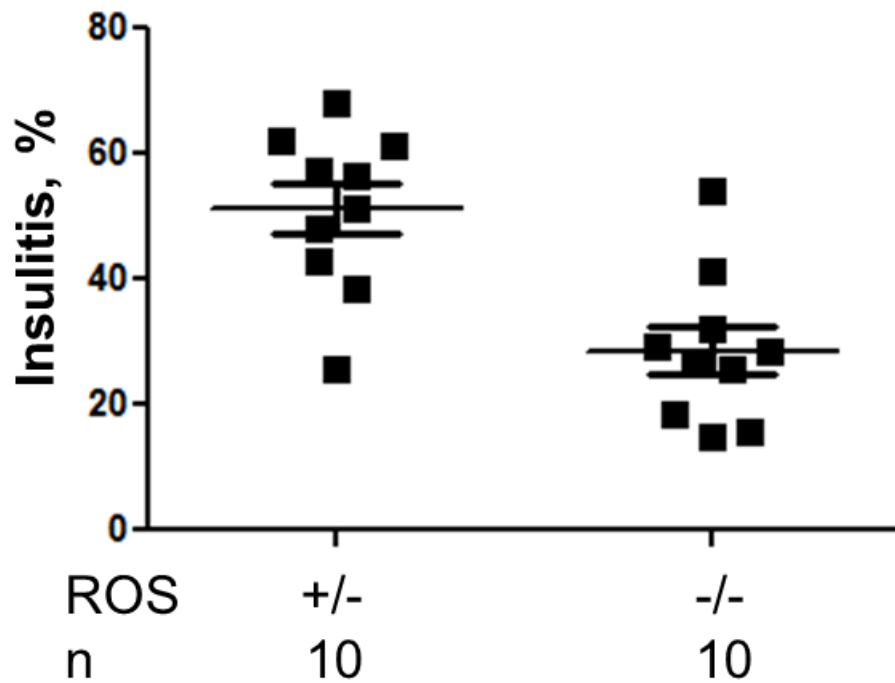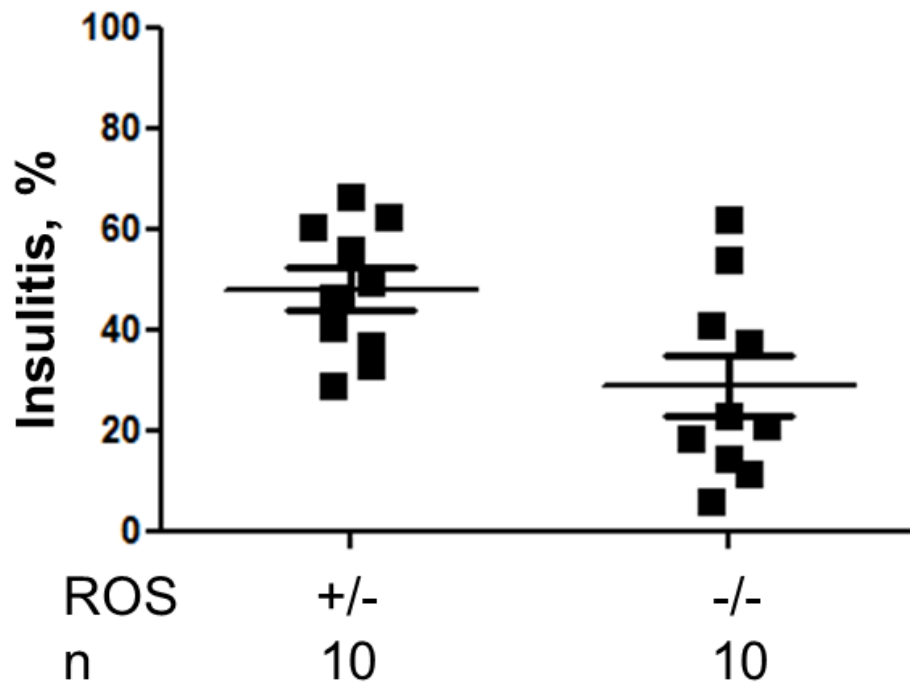**Figure 50: Insulitis observed in 30 week old GF ROS-negative NOD mice**

Bona fide insulitis (percentage of total islets) in nondiabetic 30 week old GF female

ROS-heterozygous and ROS-negative littermates. Deletion of ROS signaling rescued

insulitis compared with their ROS-heterozygous littermates (P=0.016). P values for

histopathology were determined by Student's t test. n = number of mice per group.
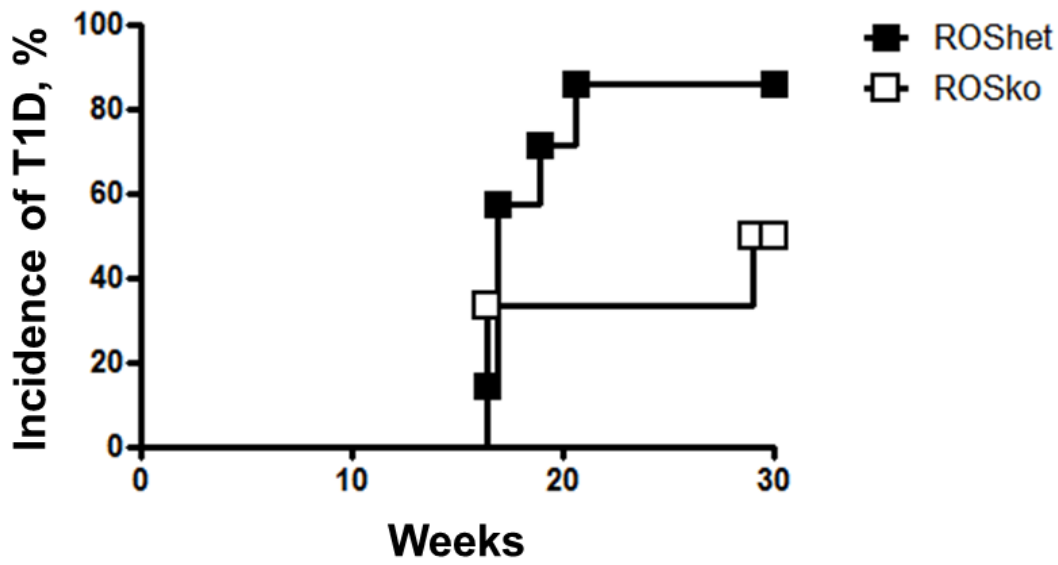
**Figure 51: T1D incidence of monocolonized *L. murinus* ROS-negative NOD mice**

Diabetes incidence in monocolonized *L. murinus* NOD female ROS-heterozygous (n=6), ROS-negative (n=7) littermates. Ablation of ROS signaling reduced incidence of disease compared with their heterozygous littermates however this decrease was not statistically significant (P=0.2276). P value was determined using Kaplan-Meier statistics.  n, number of animals per group.

GF ROS-heterozygous littermates (Figure 52).  The observation groups are not

complete, but preliminary analysis suggests that *L. murinus* alone may be capable of

rescuing T1D.

## Chapter 6: Conclusions and Future Directions

The regulation of autoimmunity by microbes may be explained by several potential mechanisms. These mechanisms include: molecular mimicry, bystander activation, or direct interference with innate immunity (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Lang et al., 2005; Malkiel et al., 2000; Rashid and Ebringer, 2012). Molecular mimicry occurs when T cells with cross-reactive T cell receptors are activated by peptides provided by the pathogen that resemble endogenous host peptides. Bystander activation would occur when adaptive autoimmunity was generated by antigen presenting cells activated by microbial stimuli and expressing self-antigens. Molecular mimicry and bystander activation both require activation of antigen presenting cells via the interaction of PRRs and their microbial ligands. Both scenarios require the presence of self-reactive T cells that have escaped deletion during thymic development. Previous work has demonstrated that splenocytes from NOD MyD88-negative mice are capable of inducing T1D upon transfer to NOD SCID mice (Wen et al., 2008). Thus it appears that islet reactive T cells are present and capable of inducing disease upon transfer to a permissive environment. However, as the initiating steps in disease have not yet been elucidated, the dissection of mechanisms by which microbes can influence autoimmunity is not a simple process. There are several levels of complexity in
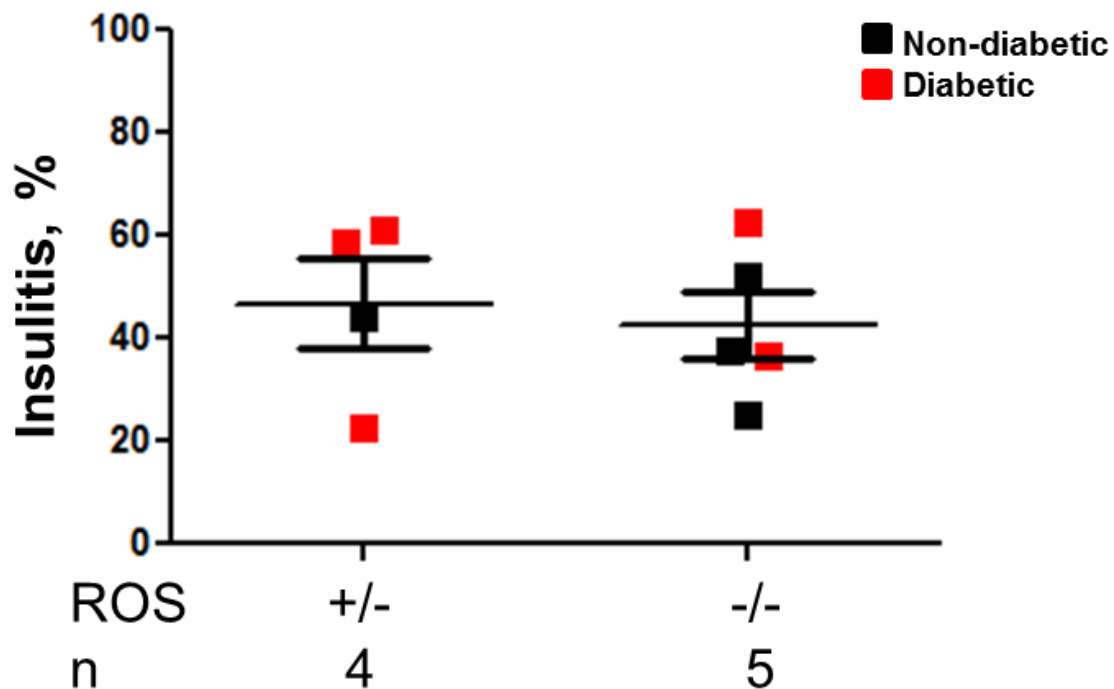
**Figure 52: Insulitis observed in 30 week old monocolonized *L. murinus* ROS-negative NOD mice**

Bona fide insulitis (percentage of total islets) in nondiabetic 30 week old monocolonized *L. murinus* female ROS-heterozygous and ROS-negative littermates. Deletion of ROS signaling did not reduce insulitis compared with their ROS-heterozygous littermates. n = number of mice per group.

designing and interpreting experiments concerning the microbiota and autoimmunity.

First, symbiosis between a host and its associated microbes are largely mutualistic. The

loss of innate control mechanisms can result in inflammation and tissue destruction

induced by otherwise beneficial microbes (Agarwal and Mayer, 2010; Shiloh et al.,

1999). These findings indicate that the host-commensal relationship is tightly regulated.

Second, the microbiota is an extremely complex community of competing organisms. It

remains difficult to identify individual lineages that are harmful or beneficial in a given

disease context. In fact, fraternities of microbes, not specific bacterial lineages, may

exert the observed regulatory functions in diabetes. Finally, intestinal microbial

communities have been demonstrated to be highly variable. Differences have been

observed between individuals of the same strain, different animal colonies, or even over

time within the same colony (Yurkovetskiy et al., 2013). Together these variables make

assigning a critical role in autoimmunity to particular lineages of bacteria extremely

challenging.

The primary goal of studying the microbiota in animal models of autoimmunity is

to discover methods to influence disease development. This could be accomplished by

either manipulation of the microbiota, or by manipulation of the signaling pathways by

which the microbiota regulates autoimmunity. Our initial efforts focused on a single

candidate microbe, SFB, which had been correlated with protection from T1D. However,

our findings were unable to either support or refute the specific lineage hypothesis as an

explanation for these results. It is important to consider that the results must be

interpreted in the context of the caveats described previously. Both monocolonization

and colonization with SFB and the full Jackson Laboratories SPF microbiota of GF NOD

mice failed to elicit protection from disease. Subsequently it was reported that transfer of monocolonized SFB into SFB negative NOD mice in one facility resulted in a level of protection from T1D.  Based on these results, and our findings, we would propose two possible hypotheses. First, that the strains of SFB used differed in their ability to induce the unknown effect that modified disease development.  Second, that there is a microbe, or fraternity of microbes, present in the Harvard animal facility that works in concert with SFB to induce protection.  Further studies to characterize the ability of the Harvard facility's microbiota and Jackson Laboratories microbiota to induce protection in the presence of SFB could be pursued.  16s sequence comparisons of the microbiota in the two facilities may reveal unique lineages responsible for the interaction with SFB.  However, this approach would face several difficulties.  First, the 16s based microbiota screening might reveal 1 unique species or it might reveal thousands. Second, the monoculture and gnotobiotic colonization of GF NOD mice with each candidate microbe isolated from Harvard's animal facility would be technically challenging. Third, this ignores the possibility that a fraternity of microbes is required, some of which are present in both facilities. These technical and experimental design considerations highlight the difficulties in studying complex systems with a biased, candidate based approach. We, as a research community, do not yet fully understand the metabolic, immunologic, anatomic, and microbial community based factors that influence individual bacterial lineages.  Therefore, attempting reductionist models with insufficient information concerning the critical variables is likely to result in divergent or contradictory findings in different facilities. Phenotypes that are not generalizable to multiple facilities can be true, but do not help us to understand the critical underlying

principles of the system we are investigating. Nor do they form a strong basis for design of potential therapeutic interventions. An alternative approach was needed.

To test the nature of the microbiota-dependent protective signal(s), we utilized a gnotobiotic approach. Colonizing GF NOD MyD88-negative mice with a variety of microbes would help us to understand how broadly the protective signals were expressed. The fact that a consortium of mouse commensals, a consortium human probiotics, and even a single bacterium was capable of providing some level of protection from disease strongly supported the balanced signal hypothesis. The groups represented highly diversified organisms in terms of metabolism, evolutionary adaptation to a host, and influence on host immune development. We hypothesized that the ability of these unrelated organisms to generate protection was a result of signaling to the host through pathogen associated molecular patterns (PAMPs).

We proposed that identification of the host pathways and activating PAMPs would be the optimal approach to address the role of microbes in T1D. The studies described here focused on testing the role of well characterized innate signaling receptors on the microbiota based regulation of autoimmunity. Our findings demonstrated a critical role for TLRs in the microbiota based regulation of T1D. Previous studies have demonstrated that both the microbiota and TLRs are not required for the development of disease, as autoimmunity develops in GF MyD88-negative NOD mice (Wen et al., 2008). This is likely a broadly applicable phenomenon, as the microbiota was also dispensable for disease in GF rat models of T1D (Rossini et al., 1979). Thus we set out to identify if the disruption of a single innate immune sensing pathway, in concert with deletion of MyD88, could restore T1D. We found that TRIF

signaling provides a protective signal in T1D. TRIF is a signaling adaptor utilized by two TLRs, TLR3 and TLR4. Thus protection is a result of activation via one or both of these receptors in response to signals from the microbiota. However, a direct assessment of the role of these TLRs has not been possible. Deletion of TLR3 or TLR4 in MyD88-negative NOD mice results in poor survival of progeny after birth. An approach utilizing cell or tissue specific conditional deletion of these TLRs could prove a viable alternative. Another alternative approach would be to compare the disease incidence of GF MyD88-negative and GF MyD88-negative TLR3 or TLR4-double-negative mice transferred to SPF conditions at weaning (four weeks of age).  This experimental approach potentially avoids the unknown factor(s) that prevent double knockouts from reaching adulthood. However, this experimental approach would bypass any early requirements for microbiota derived signals in the MyD88-negative NOD phenotype. This caveat could be controlled for by observing incidence in a control group of transferred GF MyD88-negative NOD mice at weaning.

In the absence of direct genetic experiments, there exists other data that supports a role for TLR4 in regulating autoimmunity. TLR4-negative NOD mice exhibited accelerated T1D in SPF housing conditions (Dong et al., 2012), but not as we have shown, in GF. TLR3-negative NOD mice showed no change in T1D development in SPF housing conditions (Wong et al., 2008).  We hypothesize that TLR4 signaling through TRIF is a negative regulatory pathway that protects NOD mice from T1D.  In other contexts, TLR4 has been shown to be a negative regulator of immunity against a retrovirus (Kane et al., 2011).  In that case bacterial LPS from the gut microbiota activated TLR4, driving a suppressive immune pathway that prevented an effective

antivirus response in the host. Thus the existing evidence supports a role for TLR4-mediated TRIF signaling in providing microbiota-dependent protection from T1D. However, it is interesting to note that disruption of TRIF in MyD88-negative NOD mice did not completely recapitulate the disease incidence observed in TRIF-negative NOD mice. Despite the partial restoration of overt T1D in double deficient mice, there was significant rescue of insulitis in those mice that were not diabetic at 30 weeks of age. There are several possible explanations we could posit to explain this finding. It is possible that in this genetic background the kinetics of disease development has been altered, and that observation over a longer window would see double knockout mice reach the full disease incidence, as might be suggested by the restoration of insulitis. This would be of interest as factors that influence the kinetics of disease development in the NOD mouse remain largely unknown.  Our findings could also be explained by the involvement of other microbiota-dependent negative regulatory signals that work in concert with TRIF to provide microbiota-dependent tolerizing signals. This hypothesis is supported by the observation that gnotobiotic mice lacking MyD88 were protected to various degrees by different bacterial lineages or consortia. This protection was not as complete as that seen in SPF housed MyD88-negative NOD mice. This result indicates that either specific lineages differed in their ability to activate a single protective pathway, or multiple protective pathways exist that are differentially activated by individual bacterial lineages. It would be of interest to rederive NOD MyD88/TRIF double-deficient mice into GF conditions and utilize a gnotobiotic approach to test the capacity of individual bacterial lineages to provide protection in the absence of the TRIF pathway.

In addition to the identification of the microbiota-dependent protective signaling, we also investigated the receptors upstream of MyD88 that were required to promote the disease process. We observed a MyD88-dependent disease promoting signal that required the presence of TLR2. This was only true in SPF housing conditions as GF TLR2-negative NOD mice were not protected from disease. Previous studies demonstrated a similar influence of TLR9 deletion on T1D in SPF housing conditions. In SPF housing conditions individual disruption of TLR2 and TLR 9 each produced an approximate 50% reduction in disease incidence. Thus it would be interesting to observe diabetes incidence in TLR9-negative NOD mice in SPF and GF housing conditions. This would identify if TLR9 is a MyD88-dependent, microbiota-dependent regulator of T1D. Double knockout TLR2/9 NOD mice could be observed to determine if lack of those two signaling pathways recapitulated the entire phenotype of MyD88-negative NOD mice. We hypothesize that in SPF housing conditions TLR2 and TLR9 signaling are sufficient to prevent microbiota-dependent tolerance signals and allow the development of disease. Therefore, the evidence accumulated to date identifies both pro and anti-diabetic roles for TLRs. We can conclude that TLR based sensing of the microbiota plays a regulatory rather than causative role in T1D.

Having identified microbiota sensing and signaling pathways that promote T1D, we also attempted to identify the cell populations responsible for appreciating those signals. We took the broadest possible approach, utilizing bone marrow chimeras to identify the cellular compartment that recapitulated the whole mouse MyD88-negative phenotype. However, this approach in NOD mice is burdened by a number of caveats, and we were not able to completely rule out a contribution from both the stromal and

hematopoietic compartments. Our limited interpretation of the CD45 congenic bone marrow chimera experiments was that a portion of the protection existed in radiosensitive hematopoietic cell populations. Our initial hypotheses were that the phenotype would be found in DC, as key drivers of immune responses, or gut epithelial cells, as the cells most closely in contact with the microbiota.  However, to our surprise, cell specific deletion utilizing floxed MyD88 and a cell specific Cre recombinase did not protect from T1D. Importantly, by utilizing a mixed bone marrow chimera approach, we observed protection when MyD88 was absent in B cells. NOD mice with B cells that lacked MyD88 had a 40-50% reduction in T1D incidence over 30 weeks. This level of reduction in disease incidence mirrors the observed diabetes incidence in TLR2-negative and TLR9-negative NOD mice. It is tempting to speculate that B cells may express one of the TLRs upstream of MyD88 responsible for promoting disease. Conditional deletion of the implicated TLRs in B cells is a possible approach to address this hypothesis.

Reports from Jackson Laboratories have demonstrated that NOD mice lacking B cells are highly resistant to development of T1D (NOD IgH6-negative) (Chiu et al., 2001). It was postulated that this protection could result from a critical role of B cells in T1D or from the requirement of fully developed lymphoid structures for disease to develop.  Lymphoid structure was rescued by transgenic expression of an irrelevant B cell receptor (BCR) (hen egg lysozyme, HEL) in NOD IgH6-negative. These mice were also resistant to T1D development(Silveira et al., 2002).  Thus, autoimmune diabetes required B cells which had polyclonal (likely islet reactive) BCRs. We hypothesized that the requirement for MyD88 in B cells to drive disease could reflect the requirement for B

cells in disease pathogenesis. Thus we rederived B cell knockout, and HEL-BCR rescued NOD mice into GF housing conditions. To our surprise the mice remained protected from disease. The presence of polyclonal B cell populations appeared to be a requirement for development of T1D in NOD mice, and was not specific to a role of B cells interacting with the microbiota. This was an important point, as although the protection in MyD88-negative and IgH6-negative NOD mice is very strong, they represent separate phenomena. It is therefore possible that deletion of MyD88 in B cells was mimicking the unknown disease preventing mechanism in the B cell knockout mice. To confirm that the protection observed in B cell lacking MyD88 is microbiota-dependent we have backcrossed a B cell specific Cre recombinase onto the NOD background. This will allow us to validate the results of the mixed bone marrow chimera as well as rederive NOD mice with MyD88 deleted in B cells into GF housing conditions. In GF conditions we hypothesize that Cre positive and negative littermates will both exhibit high incidences of T1D. This would demonstrate that disease promoting MyD88 signaling in B cells is the microbiota-dependent.

In addition to understanding the nature of the signaling pathways and receptors involved in regulating T1D, we also examined potential downstream effectors. Obviously MyD88 and TRIF regulate the expression of a wide variety of genes, very few of which have genetic manipulations available on the NOD mouse background. However, two effectors were of key interest: IL-10 and reactive oxygen species. We posited that IL-10 was a candidate effector cytokine in microbiota-dependent tolerance mediated by TRIF activation. We hypothesized that deletion of IL-10 alone would not alter diabetes incidence. We also hypothesized that deletion of IL-10 in MyD88-negative

NOD mice would result in rescue of T1D in the double knockouts, but not their single knockout littermates.  Surprisingly, deletion of IL-10 alone partially protected NOD mice from T1D. Additional studies from the Jackson Laboratories identified that deletion of IL-4 in addition to IL-10 in NOD mice rescues disease.  IL-4-negative NOD mice exhibit no change in diabetes incidence compared with their heterozygous littermates (Serreze et al., 2005).  Therefore the protection afforded by the loss of IL-10 may reflect a change in the CD4+ T helper profile and alter cytokine production. However, the underlying mechanism remains unclear at this stage.  Given the finding that loss of IL-10 actually protects NOD mice from autoimmunity, it is unsurprising that MyD88 IL-10 double knockout NOD mice remained completely protected from disease.  Thus we have no evidence that IL-10 is necessary for the microbiota-dependent tolerizing signals provided by TRIF.

Initial evidence for a role of ROS in microbiota dependent signaling was more promising.  Studies published at two separate facilities yielded different levels of protection from T1D of ROS-negative NOD mice.  In one facility the disease incidence was approximately 40%, at the other it was approximately 5%.  These reports lead us to hypothesize that differences in the microbiota between the facilities was influencing the level of protection afforded to ROS-negative NOD mice. Generation of ROS is a downstream component of MyD88 activation in a variety of cell types including macrophages, neutrophils, and B cells. Thus we proposed that generation of ROS was a potential downstream effector of microbiota-dependent MyD88 signaling. It was possible however that ROS-negative NOD, like B cell knockouts, represents a loss of a key process in the development of T1D.  In that instance we would expect no change in

diabetes incidence between SPF and GF housing conditions. We observed

approximately 35% incidence of disease in SPF conditions, 25% in antibiotic treated

mice, and <10% in GF conditions.  This surprising result meant that there were

microbial factors in SPF conditions that enhanced T1D incidence in ROS-negative NOD

mice.  This was the first time we had demonstrated a context where bacteria were

capable of increasing the development of disease. One possible interpretation of the

data was that we had replicated the incidence of a published report from one facility with

our SPF results, and the other facility with our GF results.  Was it possible that the

presence of specific microbes in the context of ROS-negative mice were capable of

promoting T1D?  It was clear that ROS was not simply an effector downstream of

MyD88. Deletion of ROS in NOD mice had been demonstrated to have several effects,

including: skewing CD4+ T cell responses to a Th17 phenotype and altering the

selective pressure on the microbiota (Tse et al., 2010).  To determine if a single bacteria

was capable of rescuing disease development we colonized GF NOD mice with

*Lactobacillus murinus*. Members of the genus *Lactobacillus* have been demonstrated to

be among the strongest microbial inducers of ROS. Preliminary results suggest L.

murinus is capable of rescuing T1D to a similar level observed in our SPF facilities.

Therefore, we have observed that ROS is influencing T1D in a microbiota-dependent

manner. Although, the effect does not appear simply to be a result of ROS's role as an

effector molecule downstream of MyD88 signaling.

In total, our work has demonstrated several points we feel are of interest in the

study of autoimmunity and the microbiota.  We have highlighted some of the limitations

in the design and interpretation of candidate microbe studies.  We have demonstrated

the ability of a wide variety of microbes to provide protective signals in MyD88-negative

NOD mice. This finding supports the balanced signal hypothesis.  TRIF signaling,

potentially via TLR4, was shown to provide protective signaling in the absence of

MyD88. Whereas it appears that TLR2 activation is upstream of MyD88 disease

promoting signals.  Finally, we found that MyD88 signaling in B cells promoted T1D.

These findings provide a foundation to further elucidate the mechanisms by which the

microbiota regulates autoimmunity. These results also bring us closer to the

identification of the critical targets and pathways that may be manipulated for

therapeutic intervention in this severe, chronic human disease.

# Chapter 7: Materials and Methods

**Mice**

NOD/ShiLtJ (The Jackson Laboratory, Bar Harbor, ME) mice were housed under SPF and GF conditions at the University of Chicago Animal Resource Center. TLR4negative C57BL/10ScN mice were originally purchased from the NIH and backcrossed to NOD/ShiLtJ mice for more than 10 generations. TLR2 B6 mice were a gift from S. Akira, Osaka University, Osaka, and were backcrossed to NOD/ShiLtJ for more than 10 generations. Caspase 1/11-negative NOD mice were purchased from The Jackson Laboratory. C57BL/6J-Ticam1Lps2/J mice purchased from The Jackson Laboratory and B6.129S1-Ripk2tm1Flv were backcrossed to NOD/ShiLtJ for over 10 generations and intercrossed to produce knockout and heterozygous animals for observation of diabetes incidence and histologic examination of islet infiltration. The chromosomal locations of the targeted genes were as follows: MyD88, Chr9(119335934–119341411); TRIF(Ticam1), Chr17 (56269319–56276786); Casp1, Chr9 (5298517–5307265); Tlr2, Chr3 (83836272–83841767); Tlr4, Chr4 (66827584–66930284); and Ripk2, Chr4 (16122733–16163647) according to the Mouse Genome Informatics database (www.informatics.jax.org/genes.shtml). NADPH oxidase-negative NOD mice (NOD ROS, NOD-Ncf1m1J) were purchased from Jackson Laboratories. GF animals were rederived from NOD/ShiLtJ females impregnated by TLR2-negative and TLR4-negative NOD males and kept GF at the University of Chicago. SPF mice were fed 7913 NIH-31 modified open formula mouse autoclavable diet (Harlan Laboratories). GF mice received autoclaved LabDiet 5K67. For antibiotic treated mice,

Sulfamethoxazole/Trimethoprim (Bactrim) was provided in the drinking water ad libitum at a concentration of 0.5mg/ml and 0.1mg/ml respectively. All experiments were performed in accordance with both The University of Chicago Animal Care and Use Committee and national guidelines.

**Monitoring sterility in the GF isolators**

Cecal contents or fecal pellets were collected and frozen and DNA was extracted using a bead beating/phenol – chloroform extraction protocol and amplified with universal primers that hybridize to any bacterial 16S rRNA gene sequences. Briefly, 1 fecal pellet was added to a 2 ml screw cap tube with 500 ul of 0.1 mm zirconium beads. 500 ul of a sterile filtered 2X buffer consisting of 200 mM NaCl, 200 mM Tris, and EDTA 20 mM in water was added to each sample. 210 ul of sterile filtered 20% SDS and 500 ul Phenol:Chloroform was added to each sample.  Samples were bead beaten on high for 2 minutes and centrifuged at 8,000 rpm at 4°C for 3 minutes. Aqueous phase was removed to 1.5 ml Eppendorf tube and 500 ul of Phenol:Chloroform was added. Samples were centrifuged at 13,000 rpm at 4°C for 3 minutes. Aqeuous phase was removed to a new 1.5 ml Eppendorf tube and 1/10 the volume of 3 M sodium Acetate and 1 X -20°C isopropanol  was added and the sample tube mixed by inversion.  The sample was centrifuged at 13,000 rpm at 4°C for 20 minutes. The supernatant was decanted and the sample DNA was allowed to air dry for 10 minutes and then resuspended in 1 ml water (Gibco). Tests were conducted weekly using fecal samples from individual cages. Additionally, microbiological cultures were set up with GF fecal pellets, SPF mouse fecal pellets as a positive control, sterile 1 X PBS, and sterile

culture medium controls for each sample batch. Samples were inoculated into BHI,

Nutrient, and Sabbaroud Broth tubes. Every sample in each culture media was

incubated at 37°C and 42°C in either aerobic or anaerobic environments. Cultures were

monitored daily for evidence of growth and were followed for a maximum of 5 days.

**Gnotobiotic colonization**

Gnotobiotic NOD mice were derived from GF mice by providing a specified bacterial

community via gastric gavage to the parents in a separate isolator.  Bacteria were

transferred to the progeny naturally from the mother.  The efficacy of

colonization of the progeny was tested by PCR for 16S rRNA genes specific

for the colonizing lineages. VSL3 was introduced to GF NOD mice by gavaging GF

NOD mice with VSL3 probiotic mix in 1× PBS. VSL3 mix containing Bifidobacterium

breve, B. longum, B. infantis, Lactobacillus acidophilus, L. plantarum, L. casei L.

bulgaricus and Streptococcus thermophilus was a generous gift from Dr. Claudio De

Simone (VSL Pharmaceuticals, Inc, Gaithersburg, Maryland). ASF (Dewhirst et al.,

1999) was obtained from Taconic Farms (Hudson, NY). SFB were kept frozen as cecal

contents obtained from SFB monocolonized mice, defrosted and used to colonize GF

mice.

**DiabetesTesting**

Diabetes development was monitored from 10 weeks of age by weekly testing of urine

glucose with Diastix strips (Bayer). Mice were considered diabetic following two

consecutive tests with urinary glucose concentrations over 500 mg/dL. Testing

continued until 30 weeks of age, at which point mice are euthanized and tissues collected for analysis.

**Histopathology of Diabetes**

Pancreas tissue was removed from mice and placed in Telly's fixative for a minimum of 24 hours. Pancreas tissue was placed in 70% ethanol for 24 hours prior to paraffin embedding. 5 µm tissue sections were cut using a microtome, with a 40 µm gap left between sections, and fixed to a glass slide.  Tissue slides were hematoxylin and eosin–stained and sections were analyzed for histologic scoring. The severity of islet inflammation was determined by scoring the degree of the inflammatory cellular infiltration. Briefly, at least 100 islets per group of animals were scored with pancreatic sections cut at 40-µm intervals and graded as follows: 0, no visible infiltration; I, periinsulitis; and II, bona fide insulitis. At 13 weeks of age, only the percentage of islets with bona fide insulitis was found to predict overt diabetes.

**CD45.2 Bone marrow chimeras**

To assess the role of MyD88 in hematopoietic cell populations, bone marrow chimeras were made. Bone marrow was harvested from the femur and tibia of NOD CD45.1 MyD88-heterozygous or NOD CD45.1 MyD88-negative mice. CD3+ cells were removed by anti-CD3 magnetic bead depletion as described by the manufacturer (Miltenyi Biotech). Donor bone marrow cells were mixed at a 1:1 ratio. Five week old NOD CD45.2 were subjected to irradiation (600 rads), rested for 3 hours and irradiated (600 rads). Following the second round of irradiation the mice were injected retro-orbital with

$5x10^6$ donor bone marrow cells. Mice were observed for the incidence of T1D over 30 weeks. At 30 weeks, or at the onset of T1D pancreas tissue was collected for histologic analysis.

**IgH6-negative Bone marrow chimeras**

To assess the role of MyD88 in B cell, mixed bone marrow chimeras were generated. Bone marrow was harvested from the femur and tibia of: NOD IgH6-negative, NOD MyD88-heterozygous, and NOD MyD88-negative mice. CD3+ cells were removed by anti-CD3 magnetic bead depletion as described by the manufacturer (Miltenyi Biotech). IgH6-negative bone was mixed at a 1:1 ratio with either NOD MyD88-heterozygous or NOD MyD88-negative bone marrow. Five week old NOD IgH6-negative mice were subjected to irradiation (600 Rads), rested for 3 hours and irradiated (600 Rads). Following the second round of irradiation the mice were injected retro-orbital with $5x10^6$ donor bone marrow cells. Mice were observed for the incidence of T1D over 30 weeks. At 30 weeks, or at the onset of T1D pancreas tissue was collected for histologic analysis.

**MyD88 dominance bone marrow chimeras**

To investigate whether the MyD88-negative phenotype in the IgH6-negative mixed bone marrow chimera was specific to B cells, a NOD/MyD88-negative mixed bone marrow chimera was generated. Bone marrow was collected from NOD and NOD MyD88-negative mice. CD3+ cells were removed by anti-CD3 magnetic bead depletion as described by the manufacturer (Miltenyi Biotech). NOD bone was mixed at a 1:1 ratio

with NOD MyD88-negative bone marrow. Five week old NOD CD45.2 mice were subjected to irradiation (600 rads), rested for 3 hours and irradiated (600 Rads). Following the second round of irradiation the mice were injected retro-orbital with $5 \times 10^6$ donor bone marrow cells. Mice were observed for the incidence of T1D over 30 weeks. At 30 weeks, or at the onset of T1D pancreas tissue was collected for histologic analysis.

**Statistical Analysis**

Statistical analysis of diabetes incidence was performed by Kaplan–Meier with Prism 5 (GraphPad). Statistical analysis of histology scoring was performed with Prism 5. Results are expressed as means ± SEM. The statistical difference between two groups was determined by Student's t test. For multiple groups, the statistical difference was determined with one way ANOVA. A P value of <0.05 was considered statistically significant.

**SFB quantitative PCR (qPCR) comparison**

Snap-frozen cecal content was placed in lysis buffer and transferred into autoclaved cryotube containing 500 µL of 0.1-mm zirconium/silica beads (Biospec Products, Bartlesville, OK) and 20% SDS (210 µL), bead beaten for 2 min using a Mini Beadbeater (Biospec, Bartlesville, OK), followed by phenol/chloroform extraction of DNA, as described (Turnbaugh et al., 2009). SYBR Green real-time PCR was performed using a dilution of sample that corresponded to 20 ng of original cecal content per well using SFB-specific primers: SFB736 forward 5' GACGCTGAGG

CATGAG AGCAT-3' and SFB884 reverse 5'-GACGGCACGGATTGTTATTCA-3'

(Barman et al., 2008). Cecum contents from GF mice served as negative controls.

**Flow Cytometry**

For detection of IFNγ and IL-17 in the small intestine lamina propria CD4+ T cells were

isolated.  Briefly, the small intestine was removed and washed in ice cold HBSS and

peyers patches and luminal contents removed.  Tissue was cut into 1 centimeter pieces

and placed into a 50 ml conical tube containing 40 ml 37°C CLICKs supplemented with

20 mM HEPES, 3% FBS, 5 mM EDTA and 0.145 mg/ml DTT. Tissue was placed

horizontally into an incubator-shaker at 200 rpm for 20 minutes at 37°C. Tissue pieces

were collected by forceps and transferred to a 15 ml conical containing 14 ml of CLICKs

supplemented with 20 mM HEPES and 2 mM EDTA. Samples were shaken vigorously

for 30 seconds and tissue was transferred by forceps to a fresh 15 ml conical and the

shake and wash is repeated for a total of three times. Tissue was placed into 60mm

dish containing 5 ml CLICKs supplemented with 20 mM HEPES, 0.167mg/ml

collagenase D, and 500 ug/ml DNAse. Tissue was minced with a sterile razor blade and

placed in a 15 ml conical tube and shaken at 200 rpm, 37°C for 15 minutes. Additional

10ml of CLICKs supplemented with 20 mM HEPES, 0.167 mg/ml collagenase D, and

500 ug/ml DNAse was added and placed into the incubator-shaker at 200 rpm, 37°C for

15 minutes. 10 ml of CLICKs supplemented with 20 mM HEPES, 3% FBS, and 500

ug/ml DNAse was added and tissue was strained through a 100 µm nylon filter using a

rubber syringe plunger. Tissue was rinsed with 5 ml CLICKs supplemented with 20 mM

HEPES, 3% FBS, and 500 ug/ml DNAse and centrifuged at 300 x g (1200 rpm) for 5

min. The pellet was suspended in 40 ml of 30% Percoll (Sigma-Aldrich) and centrifuged

at 700 x g (1500 rpm) for 20 minutes at room temperature with no brake. Supernatant

was removed and pellet suspended in 1 ml CLICKs supplemented with 20 mM HEPES.

Cells were transferred to a fresh 15 ml conical tube and 9 ml CLICKs supplemented

with 20 mM HEPES was added.  Cells were centrifuged at 300 x g (1200 rpm) for 5 min

and suspended in CLICKs supplemented with 5% FBS.  T Cells were stimulated with

PMA/Ionomycin for 4 hours in the presence of golgiplug (BD Biosciences).

Cells were stained with directly conjugated antibodies to CD3-Pacific Blue

(Biolegend, San Diego, CA), CD4-PE, IFNγ-APC, IL-17-FITC  (ebioscience, San Diego,

CA) utilizing the intracellular staining reagents and protocol from ebioscience.

Discrimination of dead cells was performed by staining with propidium iodide. Stained

cells were analyzed using a FACS Fortessa flowcytometer (BD Biosciences), and the

data were analyzed with FlowJo software (version 8.8.7, Tree Star, Ashland, OR).


**IgM serum ELISA**

Whole blood was collected from mice using a Pasteur pipette via the retro-orbital

venous plexus. Blood was allowed to coagulate at RT for 4 h, and overnight at 4 ˚C,

then centrifuged for 10 min at 13,000 x g. The supernatant was aliquoted into a fresh

microcentrifuge tube and centrifuged again.  The serum was frozen and stored at -80 ˚C

until use. Serum concentrations were measured by ELISA utilizing an anti-IgM capture

antibody clone eB121-15F9 (eBioscience, San Diego, CA) and HRP linked anti-IgM

detection goat anti-mouse antibody 1010-05 (Southern Biotech, Birmingham, AL).

Substrate 3,3',5,5'-Tetramethylbenzidine (Ebioscience, San Diego, CA) generates a

blue color in the presence of HRP, the reaction was stopped using 1N $H_2SO_4$. Samples were analyzed on a VersaMax microplate reader at an optical density of 450 nm (Molecular Devices, Sunnyvale, CA).

**Spleen histology**

To visualize reconstitution of the lymphoid structure, whole spleens were harvested from 12 week old NOD IgH6-negative HELtg-positive mice and placed in Telly's fixative for a minimum of 24 hours. Splenic tissue was placed in 70% ethanol for 24 hours prior to paraffin embedding. 5 µm tissue sections were cut using a microtome and fixed to a glass slide. Tissue slides were hematoxylin and eosin–stained and sections were analyzed for reconstitution of lymphoid structure.

**Scanning electron microscopy**

Small intestine was removed from gnotobiotic NOD mice and mucus was washed off using 1 X PBS. 2 mm wide, 6 mm long rectangular sections of tissue were fixed in 2% Paraformaldehyde, 2% glutaraldehyde, 0.1 M cacodylate buffer for 72 hours at 4 ˚C. The tissue was transferred to at 4˚C 0.1 M cacodylate buffer and incubated overnight. Tissue was treated with the OTOTO procedure (Malick and Wilson, 1975). In brief, tissue pieces were alternated between 1% osmium tetroxide and 1% thiocarbohydrazide solutions, washing in water between solutions. Tissue was dehydrated by passing through increasing concentrations of acetone in water (30%, 50%, 70%, 90%, and three changes of 100%), followed by hexamethyldisilazane

117

(Electron Microscopy Sciences, Hatfield, PA) and stored overnight at room temperature.

Dehydrated samples were placed on 1/8" pin mounts using colloidal silver paste

(Electron Microscopy Sciences). Mounted tissue dried overnight in a fume hood, and

tissue was subsequently sputter coated at 5 psi, at 40 mA, with the terminator set to 12

μm, and the density set to 19.8. Coated samples were imaged with an FEI Nova

NanoSEM 230 (FEI, Hillsboro, OR) at 5 kV.

**Primers**

For primers used to genotype knockout and transgenic mice in these studies see

Appendix 1: Table 1.

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**Appendix 1: Tables**

## Table 1: Mouse Genotyping Primers

| Mouse | Primers |
|---|---|
| **MyD88wt** | |
| Forward | TGG CAT GCC TCC ATC ATA GTT AACC |
| Reverse | GTC AGA AAC AAC CAC CAC CAT GC |
| **MyD88ko** | |
| Forward | TGG CAT GCC TCC ATC ATA GTT AACC |
| Reverse | ATC GCC TTC TAT CGC CTT CTT GAC G |
| | |
| **Ripk2wt** | |
| Forward | TTG GAG CTT CCT CTA GTG CTG TTC |
| Reverse | CCC AAA AAT TCA GGC TCA TTGC |
| **RIP2ko** | |
| Forward | TTG GAG CTT CCT CTA GTG CTG TTC |
| Reverse | ATC GCC TTC TAT CGC CTT CTT GAC GAG |
| **ICEwt** | |
| Forward | GAA GAG ATG TTA CAG AAG CC |
| Reverse | CAT GCC TGA ATA ATG ATC ACC |
| **ICEko** | |
| Forward | GAA GAG ATG TTA CAG AAG CC |
| Reverse | GCG CCT CCC CTA CCC GG |
| **IL-10wt** | |
| Forward | GCC TTC AGT ATA AAA GGG GGA CC |
| Reverse | GTG GGT GCA GTT ATT GTC TTC CCG |
| **Il-10ko** | |
| Forward | GCC TTC AGT ATA AAA GGG GGA CC |
| Reverse | CCT GCG TGC AAT CCA TCT TG |
| **MyD88flox** | |
| Forward | GGG AAT AAT GGC AGT CCT CTC CCA G |
| Reverse | GGA TCA TCT CCT GCA CAA ACT CG |
| **CD11cCre** | |
| Forward | TGA TGA GGT TCG CAA GAA CC |
| Reverse | CCA TGA GTG AAC GAA CCT GG |
| **CD4Cre** | |
| Forward | TGA TGA GGT TCG CAA GAA CC |
| Reverse | CCA TGA GTG AAC GAA CCT GG |
| **VillinCre** | |
| Forward | TGA TGA GGT TCG CAA GAA CC |
| Reverse | CCA TGA GTG AAC GAA CCT GG |
| **CD19Cre** | |
| Forward | CTG GTC TGG ACA CAG TGC CC |
| Reverse | CAT GCC AAG CTG GTG GCT GGA C |
| **Lys2CreCtl** | |
| Forward | CTT GGG CTG CCA GAA TTT CTC |
| Reverse | TTA CAG TCG GCC AGG CTG AC |

| | |
|---|---|
| **Lys2Cre(+)** | |
| Forward | CTT GGG CTG CCA GAA TTT CTC |
| Reverse | CCC AGA AAT GCC AGA TTA CG |
| **IgH6ko** | |
| Forward | CCG TCT AGC TTG AGC TAT TAG G |
| Reverse | TTG TGC CCA GTC ATA GCC GAA T |
| **HELctl** | |
| Forward | ACC ACA GAC CAG CAG GCA GA |
| Reverse | CTG GAG CCC TAG CCA AGG AT |
| **HELtg** | |
| Forward | ACC ACA GAC CAG CAG GCA GA |
| Reverse | GCG ACT CCA TCA CCA GCG AT |
| **CD45.1/.2** | |
| Forward | ACA AGT GCG CAG AAT ACT GG |
| Reverse | GTG CAA CGT TCA GCT TCT GA |
| **ROSko** | |
| Forward | GCC AGA AAG AAT AGC TCT GA |
| Reverse | GAC ATC TCG TCC TCT TCA AC |
| **TLR2ko** | |
| Forward | TGC ATT CCT CAG ACG CTG GAG GT |
| Reverse | TTC TCC AGC TGC TGG ACC ATG AGG |
| **TLR4wt** | |
| Forward | TTC TTC AAG CTT ACT AAC AAT TCT GTT GCT TGT |
| Reverse | AGC ACT CAG GAG GCA GAG AC |
| **TLR4ko** | |
| Forward | GTA CTT TAG GAG AGA GAG AAA GCA TG |
| Reverse | CAG GGT AAA CTG AAA AGC TGC TG |
| **Foxp3-dtr** | |
| Forward | GGG ACC ATG AAG CTG CTG CCG |
| Reverse | TCA GTG GGA ATT AGT CAT GCC |
| **TICAIM1wt** | |
| Forward | CCA GTC ATC TGA TGA CAA G |
| Reverse | CAC AGT CCA CAT AGA GGAT |
| **TICAM1ko** | |
| Forward | CCA GTC ATC TGA TGA CAA A |
| Reverse | CAC AGT CCA CAT AGA GGAT |

131