The Effect of MyD88 Expression in Dendritic Cells on Immune Function

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Abstract

Type 1 Diabetes (T1D) is a debilitating autoimmune disease that results from T-cell mediated destruction of insulin-producing β cells in the pancreas. Incidence rates of T1D are rising rapidly in the developed world for unknown reasons. It is suspected that environmental factors, especially gut microbiota, play a large role in this rise. Research has shown that certain gut microbial compositions are associated with T1D onset and that antibiotics can trigger the onset of T1D. It is still unknown how gut microbiota interact with the immune system in order to alter the development of T1D. MyD88, an adaptor protein with an important function in immune cells, has been shown to be necessary for the development of T1D in mice when gut microbiota are present in these mice. MyD88 may alter the development of T1D by modulating gut microbiota, but the mechanisms by which it does this are unclear. In this study, we examined the effect of MyD88 expression in dendritic cells, antigen-presenting cells that are important for adaptive immunity, on the concentration of TGF-β and IgA and on the levels of gene expression of various antimicrobial peptides, all of which interact with gut microbiota. We found that expression of MyD88 in dendritic cells significantly increased the concentration of TGF-β in the small intestine and significantly altered the level of gene expression of two antimicrobial peptides. This research points towards future areas of study that can elucidate how MyD88 alters the development of T1D and, ultimately, towards new treatment or prevention methods.

Review of Literature

The human body hosts a population of bacteria that is ten times greater than the population of human body cells. This collection of bacteria is known as the microbiota, and gut microbiota, which are located in the intestinal tract, have coevolved with humans to provide various benefits, including digestive support, production of nutrients, and regulation of the host immune system. The gut microbiota is traditionally thought to begin its colonization at birth, but bacteria have been found in the placenta (Satokari et al., *Microbiology,* 2009), suggesting that gut microbiota may develop *in utero*. Gut microbiota is known to be affected by both genetics and environmental factors, such as diet, antibiotics, and birth delivery mode (Jakobsson et al., *Gut,* 2014).

For many years, the relationship between gut microbiota and the human immune system has been inadequately understood. More recently, many connections between microbiota and the immune system have been identified, such as the associations between gut microbial composition and autoimmune diseases, including Type 1 Diabetes (T1D). T1D is an autoimmune disease that is characterized by T-cell-mediated destruction of insulin-producing β cells in the pancreas, resulting in insulin-deficiency and hyperglycemia. T1D is a debilitating disease due to the requirement of daily insulin injections to survive and the lack of a cure (Atkinson, *Atlas of Diabetes,* 2011). It is known that T1D is affected by both genetic factors and environmental factors due to a lack of complete concordance of T1D incidence between monozygotic twins (Ridaura et al., 2013). Deciphering the role of environmental factors in T1D development is important as the incidence of T1D is rising too rapidly to be solely attributed to genetics. In Europe, T1D incidence is increasing between 3 and 4 percent per year (Patterson et al., *Diabetologia,* 2012). While the triggers of T1D remain unknown, environmental factors appear

to be contributing to the development of T1D. Numerous studies have shown that changes in the gut microbiota correlate with alterations in the immune system and thus altered susceptibility of developing T1D (Roesch et al., 2009). Therefore, the relationship between the gut microbiota and the immune system is being investigated in individuals with T1D in order to develop new prevention methods and therapies in the future.

Gut Microbiota

The immune system requires stimulation from the gut microbiota in order to develop properly (Cheng et al., *The ISME Journal,* 2015). Consequently, it is important to understand how the gut microbiota interacts with the immune system and further, how these interactions alter susceptibility to T1D. There are over 100 trillion bacterial cells in the gut microbiota, and over 10 million unique gut microbial genes that have been identified (Whitman et al., *Proc. Nat. Acad. of Science*, 1998). In healthy humans, the majority of the gut microbiota is located in the colon (Sender et al., *PLOS Biology*, 2016) with each individual having a unique gut microbial composition (Costello, *Science,* 2009). The main commensal bacterial phyla in the gut microbiota are, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria,* and *Verrucomicrobia,* with the vast majority consisting of *Bacteroidetes* and *Firmicutes*. (Sender et al., *PLOS Biology,* 2016). There is a natural balance between the host and its microbiota. When this balance is disturbed, dysbiosis is said to occur (Marchesi et al., *Gut,* 2016). Dysbiosis has been associated with numerous chronic diseases, including obesity (Everard et al., *Proceedings of the National Archives of Science,* 2013), inflammatory bowel disease (Manichanh et, *Nat Rev Gastroeneterol Hepatol,* 2012), type 2 diabetes (Tilg al., *Gut,* 2014), and T1D (Giongo et al., *ISME,* 2011).

There are many ways in which gut microbiota can be modified, including diet, probiotics, antibiotics, and environment. Age and gender also have an effect on gut microbial composition.

Healthy pediatric gut microbiota exhibit significant compositional differences from that of an adult (Cheng et al. *The ISME Journal, 2016*) with children having an increased relative abundance of *Bifidobacterium, Faecalibacterium, and Lachnospiraceae* and with adults having an increased relative abundance of *Bacteroides.* It is currently not clear what defines a "healthy" gut microbial composition. However, a decrease in the diversity of gut microbiota is typically considered unhealthy and could potentially increase the risk for developing T1D and other autoimmune diseases (Cheng et al. *The ISME Journal, 2016*).

Animal Models of T1D

In order to get the best understanding of the development of T1D, *in vivo* studies are most frequently used. Since the number of T1D studies done in humans is limited by practical and ethical concerns, the majority of T1D research is done in animal models. The two main models for T1D research are the non-obese diabetic (NOD) mouse (Makino et al., *Jikken Dobutsu,* 1980) and the Biobreeding (BB) rat (Nakhooda et al., *Diabetes,* 1977). Both NOD mice and BB rats develop T1D spontaneously in a manner that is similar to that of human T1D. NOD mice develop T1D at about 10 weeks of age (Anderson et al., *Annu Rev Immunol,* 2005) while BB rats develop T1D from 7 to 14 weeks of age (Bortell et al., *Methods Mol Biology*, 2012). In addition to this, many of the antigens targeted by the immune cells causing the destruction of insulin-producing β cells are shared between NOD mice, BB rats, and humans (Zhang et al., *Current Opinion in Immunology,* 2008). The gut microbiota of NOD mice and BB rats is also dominated by *Bacteroidetes* and *Firmicutes* as it is in humans (Nguyen et al., 2015). One disadvantage of BB rats (over NOD mice and humans), however, is a reduced number of T cells, which are important in T1D (Pearson et al., *Journal of Autoimmunity,* 2015).

Type 1 Diabetes and Gut Microbiota

There is an abundance of research examining the relationship between gut microbiota and T1D, but there are still many questions surrounding this relationship. It is known that changes in the gut microbiota correlate with T1D, but it is still unknown which specific bacteria may cause or prevent T1D development if any do at all. Roesch and colleagues studied this relationship using the Biobreeding diabetes-prone (BB-DP) rat and the Biobreeding diabetes-resistant (BB-DR) rat. They found that bacteria belonging to the *Bacteroides* genus were more common in BB-DP rats than in BB-DR rats, while bacteria in the *Lactobacillus* and the *Bifidobacteria* genera were more common in BB-DR rats (Roesch et al., *The ISME Journal*, 2009). Another study was performed, this time using human subjects. The study, which was done by Giongo and colleagues in 2011, who examined the gut microbiota found in fecal samples of four healthy children and of four children who were susceptible to developing T1D found that the children who would later develop T1D had a higher abundance of bacteria in the *Bacteroidetes* phylum and a lower abundance of bacteria in the *Firmicutes* phylum at a young age (Giongo et al., *The ISME Journal,* 2011). Another study found similar differences in the gut microbiota between healthy children and children with T1D. There were 16 children in the case group, who had T1D, and 16 children in the healthy control group, all of which were around the age of seven and had similar exercise and dietary habits. Results showed that the gut microbial compositions of children in the control group had more similarities to one another when compared to the compositions in the case group. DNA sequencing showed that children with T1D had an increased abundance of bacteria in the *Clostridium, Bacteroides, Beillonella, Eggerthella,* and *Bacillus* genera and had decreased abundances of bacteria in the *Prevotella* and *Bifidobacterium* genera when compared to the control group. The children exhibited differences at the phylum level as well, with the children with T1D having a higher abundance of *Bacteroidetes* bacteria

and a lower abdundance of bacteria classified as *Firmicutes* and *Actinobacteria* (Murri et al., 2013).

Some studies have tried to elucidate the mechanisms of relations between gut microbiota and T1D by studying the effect of antibiotic treatment on T1D development. In a study, a combination of Sulfamethoxazole, Trimethoprim, and colistin sulfate was administered to BB-DP rats, and it was found that constant treatment from the time of weaning both delayed the onset and decreased the incidence of T1D by great amounts (Brugman et al., *Diabetologia,* 2006). Another study showed that treatment of NOD mice with Vancomycin from birth to 4 weeks of age resulted in decreased T1D incidence (Hansen et al., *Diabetologia,* 2012). However, it seems that the timing of antibiotics is important because another study found that the administration of vancomycin to pregnant NOD mouse mothers resulted in earlier T1D onset in offspring. It is unknown why this difference in timing causes such a stark difference in results, suggesting that there is still much research to be done regarding antibiotics and T1D (Hu et al., *J Autoimmuity,* 2016).

Dendritic Cells

An innate immune cell that appears to have a role in T1D development is the dendritic cell (DC). DCs are monocytes that develop in the bone marrow from a common progenitor expressing the CD1a and CD14 molecules. This progenitor differentiates into a line of immune cells that eventually become DCs. DCs are antigen-presenting cells, meaning that they collect antigens from pathogens or from host tissue and present them to naïve T cells in order to activate them and elicit an immune response or to promote tolerance, protecting the host tissue from damage. DCs are found in both lymphoid and non-lymphoid tissue and are common in mucosal areas (Male et al., 2013). DCs also have endocytic capabilities whereby immature DCs can

engulf bacteria, viruses, and host molecules and break them down into small peptides to present to T cells. Presentation of self-antigens can trigger autoimmunity when recognized by self-antigen-reactive T cells. Immature DCs initially travel to non-lymphoid tissues, and upon antigen stimulation, become active and migrate to T cell areas in lymphoid tissue to stimulate T cells and induce an immune response either by attacking pathogens directly or by secreting cytokines such as transforming growth factor beta (TGF-β). Stimulation occurs when DCs collect antigens from pathogens using MHC class II molecules, allowing them to send a variety of stimulatory molecules to naïve T cells inducing their differentiation into effector T cells, which can eliminate a pathogen (Cella et al., 1997). DCs express a collection of Toll-like receptors (TLRs), which are receptors that enable DCs to recognize pathogen-associated molecular patterns to subsequently promote an immune response (Moresco et al., 2011). A molecule involved in TLR signaling that is of particular interest in T1D research is myeloid differentiation primary response 88 (MyD88). MyD88 is an adaptor protein that works downstream of TLRs to induce the gene expression of proinflammatory cytokines to promote an immune response in response to microbial stimulation.

MyD88 and Type 1 Diabetes

A connection between MyD88 and T1D has been discovered in NOD mice. A study compared the T1D incidence of two groups of mice, which either expressed or did not express MyD88 in every cell where it could be expressed. *MyD88* knockout in specific-pathogen-free (SPF) NOD mice, which are mice that are free of certain bacteria that could interfere with an experiment, resulted in complete protection from T1D. These mice are referred to as $MyD88^{-/-}$ mice; $MyD88^{+/+}$, which are the control mice in which MyD88 is expressed in all possible cells, developed T1D. Autoimmune T cells were still present in the mesenteric lymph nodes and spleen of the $MyD88^{-/-}$ mice, meaning that the cells were prevented from entering the pancreas to cause

T1D. What is striking about this study was that the T1D development was completely restored in MyD88$^{-/-}$ mice that were reared in germ-free (GF) conditions, which are conditions that are completely free of bacteria. This suggests that the anti-diabetogenic effect of the knockout of *MyD88* relies on gut microbiota (Wen et al., *Nature,* 2008). This raised the question of how MyD88 interacts with the gut microbiota to influence T1D development. The experiments described by this paper aim to understand how MyD88 interacts with the immune system in order to alter gut microbiota and possibly affect the development of T1D.

Statement of Purpose

Our research concerns what roles MyD88 expression in CD11c^{+} DCs has on the immune responses to the gut microbiota. More specifically, we have investigated how MyD88 expression affects the concentration of IgA, an antibody found predominantly in the gut, the concentration of TGF-β, an anti-inflammatory cytokine important for making antibodies and inducing tolerance, the expression of the antimicrobial peptides crp-ductin, defcr, reg3β, reg3γ, and relmb3. We hypothesized that MyD88 expression does have an effect on the immune responses in the gut. More specifically, we hypothesized that the concentrations of IgA and TGF-β in the small intestines and the gene expression level of antimicrobial peptides would all change in response to whether MyD88 was expressed in CD11c^{+} DCs or not. Understanding these changes will help us come closer to finding a mechanism by which MyD88 interacts with the immune system and alter the development of T1D.

Methods

NOD Mice

MyD88$^{-/-}$ NOD mice and CD11c^{MyD88+} NOD mice were sacrificed at 8 weeks of age. The mice were originally obtained from the Jackson laboratory but have been bred in the Yale animal

facility for over 20 years. MyD88$^{-/-}$ NOD mice are mice that have no MyD88 expression in any cells, and CD11c^{MyD88+} NOD mice are mice that express MyD88 only in CD11c^{+} DCs.

IgA Concentration

The small intestines of the MyD88$^{-/-}$ NOD mice (n=7) and the CD11c^{MyD88+} NOD mice (n=12) were flushed with 5 mLs of sterile PBS. The flush was centrifuged at 2000 rpm for 5 minutes, and the supernatant was collected and used for ELISA (enzyme-linked immunosorbent assay) in the detection of IgA as described by Harriman et al. (1988). 96-well plates were coated with anti-mouse IgA diluted in carbonate buffer (containing 100µl of 5 µg/mL of IgA in each well). The plates were incubated at 4˚C overnight. Plates were then washed four times with 0.25% Tween 20 PBS and were incubated with 1% BSA PBS (200 µL per well) for 2 hours at 37˚C. Plates were then washed a further four times with 0.25% Tween 20 PBS. 100µl aliquots of mouse reference serum (with a known concentration) or samples from the mice, diluted in 1% BSA PBS, were added to the wells, and the plates were again incubated at 37˚C for 2 hours. Plates were washed four times again with 0.25% Tween 20 PBS and 100 µL of alkaline phosphatase substrate, p-nitrophenyl phosphate were added to each of the wells. After the substrate developed (shown by a change in color), readings were measured with a 405-nm wavelength filter. Nanograms of IgA per mL of supernatants were determined using the reference serum with a known concentration of IgA to plot a standard curve. Data were plotted with Prism™ software, which showed the mean and standard deviation, and the statistical significance was found using the Student's t test. P values less than 0.05 were considered significant.

TGF-β Concentration

The small intestines of the MyD88$^{-/-}$ NOD mice (n=8) and the CD11c^{MyD88+} NOD mice (n=12) were flushed with 5 mLs of sterile PBS. The flush was centrifuged at 2000 rpm for 5 minutes, and the supernatant was collected for ELISA for the detection of TGF-β following the manufacturers protocol (R&D systems). Data were plotted with Prism™ software, which showed the mean and standard deviation, and the statistical significance was found using the Student's t test. P values less than 0.05 were considered significant.

Antimicrobial Peptides

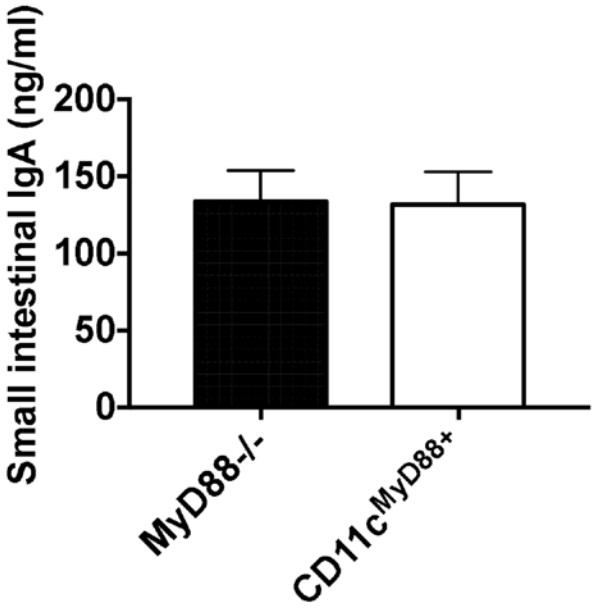
In order to measure the effect of MyD88 expression in DCs on antimicrobial peptides, gene expression levels for *C-reactive protein-ductin (crp-ductin), DEFCR-rs-10 (defcr), Reg3β, Reg3γ,* and *RELMβ (relmb)* were measured. 8-week old MyD88$^{-/-}$ (n=4) and CD11c^{MyD88+} NOD mice (n=6) were sacrificed. PBS was used to flush the distal small intestine, which was then cut into small pieces and homogenized into Trizol. RNA was extracted from the gut pieces and reverse transcribed to complimentary DNA (cDNA) with a SuperScript III First-strand synthesis kit using random Invitrogen hexamers. cDNA was used in a qPCR reaction with specific primers for *crp-ductin, defcr, reg3β, reg3γ, relmb.* Data were plotted with Prism™ software, which showed the mean and standard deviation, and the statistical significance was found using the Student's t test. After statistical analysis, P values less than 0.05 were considered significant.

Results

No Significant Difference in IgA Concentration

The concentrations of IgA in the small intestines were approximately 140 ng/mL for both MyD88$^{-/-}$ NOD mice and CD11c^{MyD88+} NOD mice, and there was no significant difference between the two (p=0.7786) as seen in Figure 1.

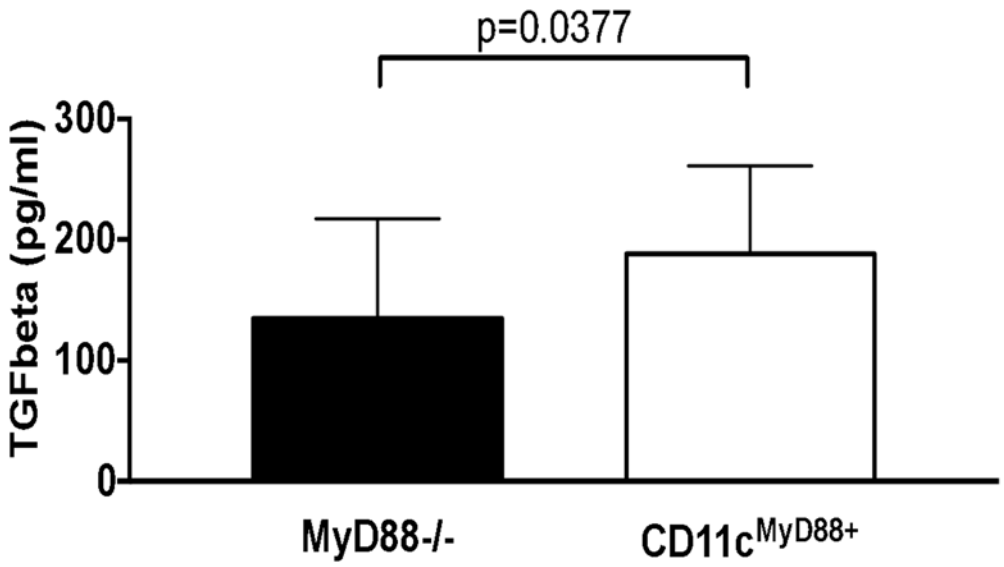
Figure 1: IgA Concentration



MyD88 Expression in DCs Significantly Increases Secretion of TGF-β

Secretion of TGF-β in the intestinal lumen significantly enhanced in *CD11c*$^{MyD88+/+}$ NOD mice compared to MyD88$^{-/-}$ mice as seen in *Figure 2.* Changes in abundance of the proinflammatory cytokines IL-17, IL-21, and IL-23 were also measured, but no significant trends were found. Thus, no data are shown for these cytokines.

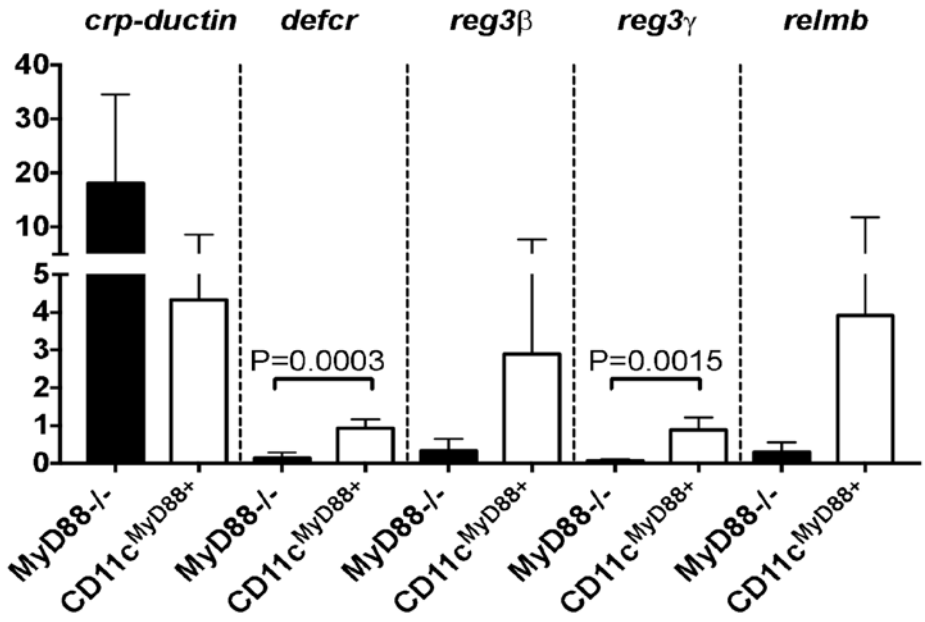
Figure 2: TGF-β Concentration



MyD88 Expression in DCs Significantly Altered Gene Expression of Antimicrobial Peptides

MyD88 significantly altered the gene expression of a number of antimicrobial peptides. CD11c^{MyD88+} NOD mice (n=6) had higher levels of expression of *defcr, reg3β, reg3γ,* and *relmb*, and CD11c^{MyD88+} mice (n=4) had lower levels of expression of *crp-ductin* as seen in Figure 3. However, only the changes of expression of *defcr* (p=0.003) and *reg3γ* (p=0.0015) were significant. The changes in expression of *crp-ductin* (p=0.0787), *reg3β* (p=0.3252), and *realmb* (p=0.3950) were insignificant, but the data for *crp-ductin* were close to the significance threshold (p=0.0787), meaning that measuring its level of expression in response to MyD88 again may be worthwhile in the future.

Figure 3: Expression of antimicrobial peptides



Discussion and Conclusions

The data help to elucidate the mechanisms through which MyD88 could possibly alter the immune system and affect the development of T1D. It is already clear that MyD88 has a role in the development of T1D (Wen et al., 2008). MyD88 in DCs is an adaptor protein that is necessary for the expression of genes relating to the secretion of proinflammatory cytokines, which can signal other cells in the immune system to react to a pathogen. These data show that overall, MyD88 has an effect on immune-gut homeostasis.

The most prevalent antibody in the gut is IgA. Deficiency of IgA has been associated with T1D. This antibody also has a role in gut homeostasis. An analysis of 150 T1D patients' medical records showed that 5.3% had been diagnosed with IgA deficiency, a figure that is 10 times the prevalence amongst the general public (Greco and Maggio, *Epidemiology*, 2015). The exact role of antibodies in T1D is still unknown and should be investigated in the future. It is somewhat surprising that MyD88 had no effect on IgA concentration since the deficiency of IgA has been associated with T1D. This suggests that the connection between IgA and T1D is not mediated by MyD88 in DCs.

It is unclear how changes in TGF-β and the antimicrobial peptides in question affect the development of T1D. Moving forward, it is important to understand the role of TGF-β and antimicrobial peptides in the immune system. TGF-β has functions in many processes, including the migration and differentiation of cells, angiogenesis, or the development of new blood cells, wound healing, and regulation of immune cells (Li et al., *Annu Rev Immunol*, 2006; Blobe et al., *N England J of Medicine,* 2000). TGF-β interacts with numerous types of cells (Herpin et al., *Developmental & Comparative Immunology,* 2004), especially with T cells. TGF-β supports the development of colonic macrophages, which, like DCs, are also antigen presenting cells that can

either induce a proinflammatory response or promote tolerance. More important, TGF-β converts naïve $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells (Esebanmen et al., *Immunol Res,* 2017). It appears that TGF-β primarily promotes tolerance in the immune system.

Since the knockout of *MyD88* completely protects from T1D development (Wen et al., *Nature,* 2008), it can be inferred that the absence of MyD88 could result in tolerogenic actions by the immune system. However, *MyD88-/-* mice in this study appeared to have lower concentrations of TGF-β, which has anti inflammatory properties. This is counterintuitive. Until additional information on the relationship between TGF-β and T1D is found, it seems that TGF-β may not be one of the mechanisms involved in the antidiabetogenic effect of *MyD88* knockout.

There is limited information on the relationship between antimicrobial peptides and T1D. However, one antimicrobial peptide, CRAMP, has been shown to protect against T1D incidence in NOD mice (Sun et al., *Immunity,* 2015). Since *MyD88* expression was found to affect levels of expression of various antimicrobial peptides, it is possible that these peptides are involved in the pathogenesis of T1D, perhaps even more so than TGF-β due to reasons discussed above. If antimicrobial peptides do have an effect on T1D development, it could be through altering gut microbiota. For example, Nod2 has been shown to affect susceptibility to T1D in NOD mice by modulating gut microbiota through secretion of antimicrobial peptides (Li et al., *Journal of Autoimmunity,* 2017). The role of antimicrobial peptides on the development of T1D should be examined further, preferably with a larger sample size, to gain a better understanding of the mechanisms involved in the antidiabetogenic effect of *MyD88* knockout since it is possible that antimicrobial peptides have an effect on T1D development.

Overall, these data show that there is much to be investigated in the future regarding immune mechanisms of the relationship between MyD88 expression and T1D. These data help

to eliminate IgA as a mediator between MyD88 and T1D and raise questions about TGF-β's potential status as a mediator. These data also open the door to further investigation of antimicrobial peptides and T1D since information on this topic is limited and has potential. Since MyD88 has been shown to have a major effect on T1D incidence, it is important to explore every possible mechanism that link MyD88 and T1D because this could eventually result in new treatments or prevention methods for T1D, which are in urgent need.

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