Modulation of the immune system by the gut microbiota in the development of Type 1 Diabetes

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Abstract

T1D is an autoimmune disease characterized by T cell-mediated destruction of insulin-producing β-cells in the pancreatic islets of Langerhans, resulting in hyperglycemia, with patients requiring lifelong insulin treatment. Many studies have shown that genetics alone are not sufficient for the increase in T1D incidence and thus other factors have been suggested to modify the disease risk. T1D incidence has sharply increased in the developed world, especially amongst youth[1]. In Europe, T1D incidence is increasing at an annual rate of 3-4%[2]. Increasing evidence shows that gut microbiota, as one of the environmental factors influencing diabetes development, play an important role in development of T1D[3]. Here, we summarize the current knowledge about the relationship between the microbiota and T1D. We also discuss the possibility of T1D prevention by changing the composition of gut microbiota.

Introduction

The elucidation of the complex interactions between the gut microbiota, metabolism, and the immune system may lead to groundbreaking changes as to how specific diseases are prevented and treated. The gut microbiota refers to the community of bacteria located within the intestine that have coevolved through millions of years with the host. This symbiotic relationship is important for many host functions including digestion, nutrient acquisition, and the development of the immune system[4].

The gut microbiota encodes trillions of genes, of which approximately 5-10 million are unique[5-7]. In total, there are ~150 times more genes than in the human genome[6]. In healthy humans, the number of the bacteria increases exponentially from the small intestine to the colon; thus, the colon is the main contributor to the total bacterial population in the gut[8]. The main commensal bacterial phlya in the gut microbiota include, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia* with the vast majority consisting of *Bacteroidetes* and *Firmicutes*.

The gut microbiota of each individual host is diverse and unique[9]. For maintenance of good health, there is a natural balance between the host and its microbial community. However, dysbiosis, which is a disturbance in the balance between the host and the microbial community, is associated with various chronic diseases, including

obesity, inflammatory bowel disease (IBD), type 1 diabetes (T1D) and type 2 diabetes (T2D)[10].

Recent evidence suggests the gut microbiota may begin colonization *in utero* as bacteria have been detected in the intrauterine environment including the amniotic fluid[11], placenta[12], meconium[13], and the umbilical cord[14]. While the majority of these studies utilize 16S rRNA sequencing or PCR, in a small number the investigators have grown bacteria by culturing the amniotic fluid[11] or the umbilical cord[14] samples in different bacterial culture conditions. In addition, the development of the gut microbiota can be influenced by the delivery mode. Infants delivered by Caesarean section (C-section) exhibit less bacterial diversity up to 2 years after birth compared with those delivered vaginally[15]. C-section deliveries have also been associated with an increased risk of obesity[16] or T1D[17] later in life, which may be linked to the fact that the gut microbiota continue to develop into adulthood[18]. This potentially provides a greater window of opportunity for therapeutic modulation of the gut microbiota.

There are many ways that the gut microbiota can be modified, including the environments we live in (e.g. rural or urban), diet and food supplements (probiotics), the use of antibiotics or other medications during illness. Other factors, such as age and gender, can also modify the microbial composition over time. For example, the healthy pediatric gut microbiota exhibit significant compositional and functional differences from those of adults[18]. It has been shown that children had increased abundances of *Bifidobacterium, Faecalibacterium, and Lachnospiraceae* compared with adults, while adults had increased abundance of *Bacteroides*[4]. Currently, it is not clear what constitutes a "healthy" gut microbial composition; however, the microbiota are indispensible for the digestion of nutrients[19], development of mucosal immunity[20], and supporting gut-brain communication[21]. Furthermore, a loss of diversity in the gut microbiota or alterations in microbial functions have been associated with risk of developing chronic diseases including T1D and other autoimmune or inflammatory disorders. Therefore, developing or retaining a "healthy" microbiota is important.

T1D is an autoimmune disease characterized by T cell-mediated destruction of insulin-producing β-cells in the pancreatic islets of Langerhans, resulting in hyperglycemia, with patients requiring lifelong insulin treatment. Many studies have

shown that genetics alone are not sufficient for the increase in T1D incidence and thus other factors have been suggested to modify the disease risk. T1D incidence has sharply increased in the developed world, especially amongst youth[1]. In Europe, T1D incidence is increasing at an annual rate of 3-4%[2]. Increasing evidence shows that gut microbiota, as one of the environmental factors influencing diabetes development, play an important role in development of T1D[3]. Here, we summarize the current knowledge about the relationship between the microbiota and T1D. We also discuss the possibility of T1D prevention by changing the composition of gut microbiota.

Animal Models of T1D

To gain the best knowledge of the mechanism(s) of a disease development, the ideal studies are *in vivo* investigations. For ethical reasons, there are considerable limitations to *in vivo* studies in humans. However, animal models of human diseases provide an alternative system to investigate the mechanism behind the immune response within the pancreas, in the case of T1D, or the bacteria in the gut, *in vivo*, to answer questions that we cannot do in humans. Utilizing bench to bedside and bedside to bench approaches can further expand our understanding and help us to achieve the ultimate goal of preventing T1D development or to develop a cure.

There are two widely used rodent models for human T1D research - the non-obese diabetic (NOD) mouse[22] and the bio-breeding (BB) rat[23]. Both the NOD mouse and the BB rat develop spontaneous T1D, similar to humans. NOD mice usually develop T1D after 10 weeks of age[24], while BB rats develop T1D from 7 to 14 weeks[25]. In affected humans, the age of onset of T1D typically ranges from 6 months to late adolescence. The NOD mouse and the BB rat also carry the T1D susceptibility major histocompatibility complex (MHC) class II genes, similar to the human T1D susceptibility MHCII alleles. The NOD mouse recapitulates many features of human T1D, especially those T1D susceptibility genes[26]. However, the pathogenesis of T1D in both humans and animal models is not solely determined by genetics; the disease onset is influenced by a combination of genetic and environmental factors. Pathologically, humans[27], the BB rat[28] and NOD mice[29] display infiltration of lymphocytes in the pancreas, namely, insulitis. The immune cells that are involved in the destruction of

insulin-producing beta cells in humans, are similar to those cells present in NOD mice and BB rats during diabetes development. These cells are mainly autoreactive T cells as T1D is a T cell-mediated disease. Furthermore, the autoantigens that the T cell recognizes in human T1D are also present in the NOD mouse[30]. In humans, a gender bias emerges after puberty with a small increase in the number of affected males[31], while the majority of diabetic NOD mice are females[22]. However, there is no gender bias in T1D development in BB rats[23].

Unlike human studies, studies using animal models can be better controlled in order to minimize variables and assess the effect of different environmental factors, such as diet, mode of birth delivery and usage of medication, on the gut microbiota and the development of T1D. Therefore, studies using animal models provide an extremely valuable and unique tool for gaining more insightful knowledge about the disease. In addition, humans[32] and rodents[33, 34] with T1D have been shown to exhibit similar gastroenterological abnormalities, including increased intestinal permeability, altered microvilli, leaky tight junctions, and altered gut microbiota[35-37]. Similar to humans, *Bacteroidetes* and *Firmicutes* are also the dominant phyla in relation to the composition of gut microbiota. However, there are also major differences as 85% of the bacterial genera found in mice are absent in humans[38]. Moreover, a disadvantage of well-controlled studies using in-bred animal models may be a lack of direct translation to humans, who are extremely heterogeneous.

Gut Microbiota and T1D

Hygiene Hypothesis

The concept of the gut microbiota as a major environmental factor influencing T1D supports the rising incidence rates in developed countries. The hygiene hypothesis originally was coined in relation to observations of respiratory problems, hygiene and household size[39]. A modification of this may help to explain the increased T1D incidence as a result of reduced diversity in the microbiota. The sharp increase in T1D incidence dates back to the mid 20th century where children were raised in environments with increased levels of sanitation and thus have less exposure to bacteria and parasites.

The hygiene hypothesis has been tested in NOD mice, as the cleaner the living conditions, the higher the incidence of diabetes found in NOD mice[40]. Moreover, studies have found that infection of NOD mice early in life with a number of different bacteria can prevent T1D[41, 42]. Human epidemiological studies showed that the incidence of T1D and allergies is much lower in developing countries where the living standard is low but the rate of bacterial or parasite infection is high[43]. Links found between gut microbiota and T1D, discussed in this review, have prompted questions on how the gut microbiota can be modulated in order to alter T1D development.

Gut Microbiota in T1D

Both the gut microbiota composition and the immune system co-evolve and develop together over time, and young children have reduced microbial diversity and a less mature immune system compared to adults[18, 44]. Therefore, it is important to understand how the gut microbiota interacts with the immune system and further, how these interactions alter susceptibility to T1D. Roesch and colleagues found that bacteria of the *Bacteroides* genus were more common in diabetes-prone BB rats (BB-DP) than they were in diabetes-resistant BB rats (BB-DR)[45]. However, the abundance of bacteria belonging to the *Lactobacillus* and *Bifidobacterium* genera was higher in BB-DR rats than in BB-DP rats[45]. Altered microbiota were also found between the NOD mouse and non-obese diabetes resistant (NOR) mouse[46]. According to Daft and colleagues, the NOD mouse has a lower *Firmicutes:Bacteroidetes* ratio as well as a lower abundance of *Prevotella* compared to the NOR mouse[46]. This profile is also seen in children with T1D compared to age-matched healthy children[37]. Long-term changes in the gut microbiota of NOD or NOR mice can be accomplished by cross-fostering, whereby NOD mice are nursed by NOR mothers and vice versa[46]. Cross-fostering of NOD mice results in both the loss of some diabetogenic bacteria and the gain of bacteria associated with diabetes protection. Further, NOD mice fostered by NOR mothers had a decreased incidence of T1D[46].

Gut microbiota can modulate T1D susceptibility associated with known T1D susceptibility loci. Genetic susceptibility at the MHC loci is the most important risk factor for T1D development in both NOD mice and humans. NOD mice express the gene

encoding MHC class II IA^{g7}, which is a homolog of human HLA-DQ8[47-49]. NOD mice do not express IE, another MHC class II gene, a homolog of human HLA-DR[48, 49]. Expression of IE, or expression of IA^{b}, the MHC class II locus for C57BL/6 mice, instead of IA^{g7}, is associated with protection from disease in NOD mice[50, 51]. Interestingly, a recent study by Silverman *et al.* suggested the mechanism of disease protection is mediated by gut microbiota in IE-expressing NOD mice[52]. The authors showed that expression of the IE transgene results in the compositional changes in gut microbiota which contributes to T1D protection. To prove that the disease protection was indeed mediated by the gut microbiota, the authors treated the IE transgenic NOD mice with different antibiotics. Administration of vancomycin or metronidazole, but not neomycin or ampicillin, in the drinking water, disturbed the gut microbiota sufficiently to induce insulitis in the normally insulitis-free IE transgenic NOD mice[52]. However, although the presence of IA^{b} in NOD mice was also able to alter the gut microbiota composition, the effect on diabetes development was minimal[53]. Expression of other T1D protective genetic loci *Idd3* and/or *Idd5* from C57BL/6 mice in NOD mice[54, 55] did not lead to changes in gut microbiota but enhanced IL-2 production and Treg function. Mullaney and co-authors further assessed the microbiota from healthy humans carrying the *Idd3/5* protective alleles and found similar gut microbiota composition to NOD mice expressing the same alleles[53]. These studies not only support the importance of the NOD mouse model of human T1D, but also reveal the importance of the genetic susceptibility loci in T1D, which modulate the interactions of immune cells and gut microbiota.

Alterations in the gut microbiota have also been observed in humans with T1D. A study by Giongo and colleagues analyzed bacteria in fecal samples of infants and young children and discovered that children who developed T1D had higher proportions of bacteria from the *Firmicutes* phylum and lower proportions of bacteria in the *Bacteroidetes* phylum than age-matched healthy controls at 4-8 months of age[37]. However, by the age of 2, children who had developed T1D had a higher proportion of *Bacteroidetes* and a lower proportion of *Firmicutes* relative to healthy controls[56]. Rather than using stool samples, one study compared the duodenal gut microbiota of patients with T1D, or those with celiac disease (CD) and with that of the healthy control subjects[32] (due to proximity of the duodenum and close relationship to the pancreas).

Some patients with T1D in the study had a gastroduodenal endoscopy and biopsy for CD diagnostic purposes. However, the authors found a distinctive inflammatory profile in the patients with T1D[32]. Patients with T1D showed overexpression of ten inflammation-associated genes in the biopsies, including chemokines and TNFα, compared to both healthy controls and CD patients[32]. Further, only patients with T1D exhibited an increased *Firmicutes* and *Firmicutes/Bacteroidetes* ratio but reduced proportion of *Proteobacteria* compared to either patients with CD or healthy controls[32].

In addition, some studies have investigated the gut microbiota in individuals who are positive for islet autoantibodies and those who are not. In a US-based study, the gut microbiota composition was found to be different between seropositive individuals and their seronegative first-degree relatives (FDRs) with an increased abundance of *Catenibacterium*, *Prevotellaceae* and *RC9 gut group* bacteria in the former[57]. Interestingly, the authors also found that the overall composition of gut microbiota in autoantibody-positive individuals and seronegative FDRs were similar but different from those recent-onset T1D patients and unrelated healthy controls[57]. It is not clear if the FDRs were living in the same or a similar environment; however, this suggests some genetic influence in the composition of gut microbiota and changes in the gut microbiota prior to and/or soon after T1D development. In a European study, Endesfelder and coauthors also compared the compostion of gut microbiota between seropositive or seronegative individuals who have an FDR with T1D[58]. Their results did not reveal any differences between autoantibody-positive and -negative individuals in microbiota diversity and composition, as well as single-genus abundance[58]. However, the authors found substantial changes in microbial interaction networks, especially in young children who later developed autoantibodies[58]. In another study, the microbiota composition and alpha diversity in European children, who had seroconverted and later developed diabetes, was different to those who did not seroconvert[56]. In addition, the children who seroconverted but had not developed diabetes by 3 years of age, had a microbiota composition and alpha diversity more similar to non-seroconverters. Together, the data confirmed microbial changes prior to and post-diabetes development. While most studies have focused on 16S rRNA sequencing of the microbiota, Pinto and colleagues investigated the microbial proteome, i.e. the proteins expressed by microbiota isolated

from stool samples of healthy children and children with T1D[59]. The authors found that children with T1D had a higher abundance of proteins from *Clostridia* and *Bacteroidetes*, while healthy children had a higher proportion of proteins from *Bifidobacterium*. Although many studies provide evidence of altered gut microbiota in individuals with T1D compared to healthy control subjects, few have shown a causal link between the altered gut microbiota and the disease. However, it is important to note that some of the human studies had very small group sizes, with as few as 3-4 individuals/group[37, 59]. Thus, larger studies are needed. However, the data from the current human studies suggest that the altered microbiota and their interactions with the immune system are likely to contribute to T1D susceptibility. Therefore, it is important to do functional studies *in vivo* and animal models can provide the perfect tools for this purpose.

Pattern Recognition Receptors

Pattern recognition receptors (PRRs) are germ-line encoded and thus have been conserved over thousands of years to bind conserved structures from pathogens, designated as pathogen-associated molecular patterns (PAMPs), which are present in microorganisms[60]. An example of a PAMP is lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria[61]. There are several PRR families including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs).

TLRs

At least 10 and 13 members of the TLR family have been identified in human and mouse, respectively, all of which recognize different PAMPs[62]. TLRs are expressed either on the cell surface (TLR1, 2, 4, 5, 6, 10, 11 and 12) or within the cells (TLR3, 7, 8, 9 and 13). TLRs function via signaling through the activation of the NF-κB signaling pathway, inducing proinflammatory cytokines that enable the innate immune system to quickly eradicate potential microbial threats.

There are two major TLR signaling pathways mediated by different adaptor proteins, the Myeloid differentiation primary response gene 88 (MyD88) and the Tir-domain-containing adaptor-inducing interferon β protein (TRIF). TLR3, a receptor for

double stranded RNA, relies solely on TRIF signaling, while TLR4, a receptor for LPS, can signal through both MyD88 and TRIF; all the other TLRs rely on MyD88[63-65]. Antigen presenting cells (APCs) express many TLRs and play an important role in linking gut microbiota and the host immune system[66]. TLR signaling is essential because it enables optimal antigen presentation by inducing APC maturation and costimulation, as well as the release of cytokines[61, 65, 67-69].

TLR interactions with the gut microbiota have been found to be important in contributing to T1D susceptibility. MyD88-deficient (*MyD88*$^{-/-}$) NOD mice are completely protected from diabetes under normal, specific-pathogen free (SPF) conditions, where gut microbiota are present[70]. However, diabetes was partially restored in the *MyD88*$^{-/-}$NOD mice after antibiotic administration, suggestive of microbial involvement in the protection of the mice. Interestingly, re-deriving the *MyD88*$^{-/-}$NOD mice to germ-free (GF) conditions abolished the disease protection. Introduction of commensal bacteria to the GF *MyD88*$^{-/-}$NOD mice markedly reduced diabetes development. These results demonstrate that MyD88-dependent signaling is important for T1D development, which can be modulated by gut microbiota. Burrows and colleagues reported that diabetes susceptibility in *TLR4*$^{-/-}$NOD mice and protection in *TLR2*$^{-/-}$NOD mice were also modulated by gut microbiota[71]. LPS recognition has recently drawn more attention in the T1D research field. It is known that Finland has the highest T1D prevalence worldwide, whereas the incidence of T1D in Russia Karelia, a close neighbor of Finland, is six times lower[72]. In addition, there is a greatly reduced risk of developing other autoimmune and allergic diseases in Russion Karelia[73, 74]. Investigation of the microbiota composition in children living in the different regions revealed that Finnish children had more *Bacteroides* species encoding more LPS synthesis genes, when compared to Russian infants[3]. Furthermore, the LPS from the *Bacteroides* species isolated from Finnish children was structurally and functionally different from the LPS of *E.coli,* a *Bacteroides* species, found in Russian infants. When the immune function of the two different LPS types in NOD mice was tested, the LPS from *Bacteroides* isolate of the Finnish children was more immunostimulatory than the LPS from the *E.coli* isolate from the Russian children. The finding suggests that altered LPS recognition by TLR4 may be important in modulating susceptibility to T1D.

Interestingly, TLR3-deficiency on the NOD background had no impact on diabetes development; however, viral infection models of diabetes development required TLR3 on both the NOD background as well as other genetic backgrounds for diabetes to develop[75-79]. Furthermore, it was noted that enhanced costimulatory molecule expression in the islets, using transgenic constructs directed by the rat insulin promoter, involved changes to the gut microbiota and signaling through TLR3 and MyD88 pathways[79]. Our studies also showed that TLR9-deficient NOD mice[80] and TRIF-deficient NOD mice[81] are protected from T1D development. LPS is the ligand of TLR4 and TRIF is one of the two downstream signaling pathways of TLR4. It is interesting that diabetes protection in TRIF-deficient NOD mice is mediated by gut microbiota[81]. Taken together, most TLRs are required for T1D development in NOD mice, while TLR4 signaling regulates the development of T1D through gut microbiota and/or LPS.

NLRs

Another family of PRR is the nucleotide-binding oligomerization domain-like receptors (NLRs). These NLRs recognize both PAMPs and damage-associated molecular patterns (DAMPs; molecules produced by stressed cells to promote an inflammatory response). One of the best-studied NLRs is the nucleotide-binding oligomerization domain-containing protein 2 (Nod2). Mutations in this receptor mediate susceptibility to inflammatory bowel disease in humans[82, 83]. We recently showed that Nod2 also influences T1D development in NOD mice, mediated by altered gut microbiota[84]. In this study, *Nod2*$^{-/-}$NOD mice were protected from T1D only when housed with other *Nod2*$^{-/-}$NOD mice. If the *Nod2*$^{-/-}$NOD mice were housed with Nod2-sufficient wild-type NOD mice, the *Nod2*$^{-/-}$NOD mice developed a similar T1D incidence to WT NOD mice. This provides important evidence that the environmental conditions e.g. housing status can alter the interpretation of the disease phenotype in genetically modified mouse strains in T1D studies.

The nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3 (NLRP3) inflammasome is another protein involved in the detection of pathogens by innate immune cells. We found reduced expression of chemokines and chemokine receptors on both pancreatic islets and T cells in *NLRP3*$^{-/-}$NOD mice, which

protected the mice from developing T1D[85]. It is currently unknown whether this process is modulated by the gut microbiota. The role of other NLR members in mediating T1D susceptibility is also unknown.

APC and gut microbiota interactions

Dendritic cells (DCs)

DCs are potent APCs found in both lymphoid and non-lymphoid tissue. Studies have shown that DCs are enriched at mucosal sites[86, 87] and can sample microbial antigens from the gut lumen, presenting the antigens to the T cells and possibly B cells, residing in the mucosal tissue[88, 89]. Mucosal DCs express a number of chemokine receptors and tissue homing adhesion molecules including CD103, all of which facilitate the intestinal DCs to migrate to other tissue sites after antigen uptake from the gut lumen[90, 91]. DCs express a range of PRRs, and signaling through these induces maturation, promotes survival and maintains homeostasis within the DCs[69, 92-98]. Thus, DCs are a pivotal component of the immune system, linking innate and adaptive immunity and inducing a specific immune response to pathogens but also to self-antigens in the context of autoimmunity.

In T1D, DCs present self-antigen and activate autoreactive T cells which damage and destroy pancreatic β-cells[99]. DC activation through TLR9 and/or TLR3 has been shown to be important in enhancing IFNα secretion and thus promoting T cell activation and T1D development in NOD mice and humans[100, 101]. Turley and colleagues have also shown that pancreatic draining lymph nodes (PLNs) function as an intersection for immune responses to gut non-self antigen and pancreatic self-antigen[102]. It is conceivable that some microbial antigens may share antigenic homology with pancreatic self-antigen, which can be sampled by intestinal DCs and promote undesirable autoimmune responses. In this regard, we have recently shown that autoreactive and diabetogenic CD8 T cells recognize a microbial peptide and promote the acceleration of T1D development in a T cell receptor (TCR) transgenic NOD mouse[103].

B cells

B cells originate and develop in the bone marrow. Mature B cells can differentiate to antibody-secreting plasma cells. IgM is the first antibody that B cells produce in response to pathogen invasion. However, IgM is usually low affinity and hence the need for affinity maturation including class switching, i.e., Ig gene rearrangements, to generate antibodies with high affinity, in order to more specifically target the antigen and control pathogen invasion[104-106]. B cells undergo class switching in response to different antigens. The type of class-switched antibody produced by B cells is also determined by the tissue location. For example, IgA is the predominant antibody in the mucosal tissues, while IgG is the most prevalent antibody in the circulation[107]. B cells often require T cell help to complete class switching; however, commensal bacteria and/or bacterial antigens presented by mucosal DCs can induce B cells to secrete IgA, which protects the mucosa from invasion by pathogens and reduces inflammatory signals[108, 109]. Intestinal epithelial cells can also induce IgA secretion[110].

In addition to antibody secretion, B cells also function as antigen presenting cells, promoting diabetes development[111-113]. Although T1D is considered a T-cell mediated disease, it has become clear that B cells play an important role in the development of T1D. The precise role of B cells in the pathogenesis of T1D is complex and not fully understood; however, the autoantibodies produced by self-reactive B cells provide a very good biomarker to predict T1D onset in the individuals with high risk of developing T1D. The self-reactive B cells in T1D produce IgG autoantibodies to a range of antigens including insulin[114], GAD[115], IA-2[116] and ZnT8[117]. Less is known about the role of IgA in T1D, although data from two T1D studies provide conflicting evidence as to whether the IgA concentrations in the serum are different between controls and T1D patients[118, 119]. IgA is enriched in mucosal sites of both mouse and human in a secretory form and it is estimated that approximately 40 mg/kg body weight of IgA are produced in human intestine[120, 121]. Detection of immunoglobulins bound to the bacteria within the intestine[122, 123] may provide new avenues of investigation for T1D studies, enabling us to understand which gut microbiota become antibody targets and how the immune system interacts with them.

B cells in NOD mice express enhanced levels of some PRRs (and associated molecules) compared to C57BL/6 mice[124]. Further, unmethylated CpG

deoxyoligonucleotides, which are a ligand for TLR9, activated pro-B cells. These cells have a regulatory function and are capable of protecting NOD mice from T1D development[125]. Interestingly, the C1858T (lyp) polymorphism of the PTPN22 gene in humans was also associated with altered B cell responses in individuals with T1D after stimulation with CpG[126]. Therefore, innate signals can mediate important changes altering susceptibility to T1D in both NOD mice and humans. However, B cells are not the only adaptive immune cells to be influenced by the gut microbiota.

T Cells are influenced by gut microbiota

T cells are a major player in the adaptive immune responses that both fight against pathogens and regulate immune responses to maintain immuno-homeostasis. T cells originate from bone marrow stem cells and undergo development in the thymus (including the gene rearrangements that enable the antigen-specific TCR to be expressed). Thymic T cells also undergo selection processes, ensuring the deletion of highly auto-reactive T cells, which prevents autoimmunity. However, this process is not complete and may contribute to the development of autoimmune disorders in people who are susceptible to autoimmunity.

T cells expressing their antigen-specific TCR, can recognize a vast diversity of antigens, pathogens and non-pathogens, which include self-antigens[127]. However, the antigens recognized by the TCR are presented by APCs through the antigenic peptide-MHC complex. The APCs express costimulatory molecules and produce cytokines, and these, together with the recognition of specific antigen, stimulate T cells to differentiate into different effector subsets. Studies have shown that commensal bacteria, most likely mediated by APCs, can induce T helper (Th) 1[128, 129], Th2[130, 131], Th17[132, 133] and T follicular helper (Tfh) cells[134]. For example, the presence of Segmented Filamentous bacteria (SFB) in the gut can induce IL17-producing CD4+ Th17 cells [132, 133], and Tfh cells[134]. Targeting SFB specifically may enable potential treatments for Th17-driven autoimmune diseases e.g. Experimental Autoimmune Encephalomyelitis (EAE, a mouse model of multiple sclerosis) and collagen-induced arthritis in mice[135, 136]. Although, T1D is driven by Th1 cells, SFB have been shown to protect NOD mice from T1D when housed in SPF conditions[137]. However, SFB introduction into GF NOD mice had no

effect on T1D development in female mice, whereas there was a significant delay in T1D onset in male GF mice after introducing SFB[138]. Other studies have shown the inter-regulatory relationship between Th1 and Th17, where Th17 cells are also controlled by Treg cells. It is plausible that the Th1/Th17/Treg axis plays an important role in modulating T1D susceptibility by alteration of gut microbiota. The newly-identified role of SFB in altering Tfh differentiation and trafficking in a mouse model of arthritis is interesting[134]. This provides evidence of the microbiota altering T:B cell interactions within the germinal centers prior to autoimmunity development. In T1D, Tfh T cells from diabetic NOD mice can transfer diabetes[139] and Tfh cells were found to be increased in T1D patients[140]. A recent study investigating insulin-specific T:B cell interactions in NOD mice, also revealed an increase in Tfh cells associated with increased diabetes development[141]. Therefore further understanding antigen-specific germinal center interactions and the influence of gut microbiota, prior to autoimmunity development may be very important.

Treg cells

Tregs are characterized by their expression of the forkhead box transcription factor (FoxP3)[142], and are potent at suppressing immune responses[143]. Tregs were previously named suppressor T cells[144], and we have gained much more understanding of these cells in recent years. Tregs can be generated within the thymus (natural Tregs, nTregs) or in the periphery (induced Treg, iTregs). FoxP3 deficiency, caused by the Scurfy x-linked mutation[145], resulted in severe immune cell infiltration in multiple organs and autoimmune destruction[146, 147]. Thus, Treg cells are vital in mediating immune tolerance to autoantigens. In addition, they are also very important in limiting the immune response to foreign antigens to prevent tissue damage.

Tregs, like other immune cells, also express TLRs including TLR4, TLR5, TLR7 and TLR8 as identified by real time PCR[148]. Upon LPS stimulation, that is recognized via TLR4, Tregs became more activated and exhibit enhanced suppressive capabilities[148]. Studies have shown that commensal bacteria, such as strains of *Clostridium,* by promoting a TGF-β rich environment, induce T effectors to become Tregs in the colon and protect mice from chemical induced colitis[149]. Treg induction was also confirmed in

mice given a mixture of human stool-isolated *Clostridium* species[150]. Interestingly, Treg induction by *Clostridium* species was independent of PRR signaling, as Treg induction was not impaired in a number of PRR deficient mice including *MyD88*$^{-/-}$ mice[149]. However, other studies have shown that Treg conversion in colonized germ-free mice with the altered Schaedler flora (ASF, a mixture of 8 strains of human gut bacteria including a *Clostridium* species) is MyD88 dependent[151]. It is not clear whether other strains of bacteria in the ASF also contribute to the Treg conversion. However, these studies suggest that Treg induction and the mechanism behind it depend on the type of bacteria. More recently, Nod2, a member of the NLR family, which recognizes the bacterial component muramyl dipeptide (MDP) was also found to regulate human Treg survival by preventing apoptosis induced by MDP stimulation; however, Tregs from patients with IBD, who have the Nod2 gene mutation, were not protected from apoptosis[152]. We have shown recently that Nod2-deficient NOD mice are protected from T1D development, which was, at least in part, mediated by increased gut microbiota-induced Tregs in the pancreatic lymph nodes[84]. This data shows the microbiota and immune recognition together shape the regulatory T cell response and may provide an important target for therapy.

Metabolism and the gut microbiota

The intestinal microbiota utilize undigested food products as substrates for fermentation resulting in the production of different metabolites. Short-chain fatty acids (SCFAs), saturated fats, L-carnitine, and choline are examples of microbially-derived metabolites. Therefore, the presence of these metabolites depends on the microbiota composition. Studies have shown that the metabolites, especially SCFAs influence the differentiation and function of immune cells; thus, they may play an important role in the development of T1D.

Other metabolic components including sex hormones can affect the immune system including the induction of autoimmunity. The female gender bias in T1D development seen in the NOD mice has been shown to be consequent upon interactions between sex hormones and gut microbiota[138, 153]. The gut microbiota in male NOD mice influence the levels of testosterone, and higher levels of testosterone are associated with

the protection against T1D development[153]. Several human autoimmune disorders have a strong gender bias, with a higher incidence in women. While human T1D in adulthood has a small gender bias towards men, it is important to decipher the role(s) of hormones and microbiota including microbial products in mediating susceptibility to autoimmune diseases.

Metabolites

One of the most significant metabolites produced by gut microbiota are SCFAs, during the fermentation of dietary fiber in the colon. Butyrate, propionate, and acetate are the major SCFAs produced in the gut, which can regulate the host immune system, central nervous system, gastrointestinal system, and metabolism through various mechanisms. The oxidation of SCFAs, butyrate in particular, is the principal energy source utilized by colonocytes[154, 155]. Butyrate also enhances the integrity of human and mouse intestinal epithelial cells. This occurs by controlling the assembly of tight junctions as demonstrated in an *in vitro* culture system[156] and in mice *in vivo* through the stabilization of the hypoxia-inducible factor (HIF), a transcription factor important for mediating epithelial barrier functions[157]. Moreover, butyrate can be sensed by the immune system to promote Treg induction, concomitant with decreased inflammation in the intestines[158-160].

The short chain fatty acid propionate is converted into glucose in the intestine, resulting in decreased glucose production from the liver[161]. Propionate also acts as an agonist of FFAR3, inducing the peripheral nervous system to alter host metabolism by decreasing adiposity, body weight and glucose production in the liver, thus promoting better glucose control[162]. In addition, SCFAs can regulate the expression of peptide YY (an enteroendocrine hormone) that controls gut motility and transit rate, as well as SCFA uptake and can promote anti-inflammatory properties, offering protection from induced inflammatory diseases such as colitis, arthritis and asthma in mouse models[163, 164]. Thus, increased production of SCFAs from dietary fiber supplements or the ingestion of probiotics can inhibit pro-inflammatory cytokines and chemokines that could be used in potential treatments for autoimmunity e.g. colitis. However, a balance would be

required, as SCFAs are an additional source of calories and can be associated with obesity and metabolic syndrome[165].

While less is known about the role of SCFAs in the development of T1D, a few recent studies have suggested that SCFAs can modulate T1D susceptibility. One study showed that feeding NOD mice with acetylated or butyrated high-amylose maize starch diets increased their serum concentrations of acetate or butyrate and protected NOD mice from developing diabetes[166]. Further, protection was enhanced if NOD mice were fed with a combination of both acetylated and butyrated diets. Interestingly, NOD mice fed with the high acetate diet had reduced frequencies and numbers of islet autoantigen-reactive T cells in the spleen and pancreatic lymph nodes (PLNs). However, the protection in NOD mice fed with a high butyrate-containing diet was related to increased Treg number with enhanced suppressive functions[166]. Another study demonstrated that butyrate could influence the secretion of cathelicidin-related antimicrobial peptide (CRAMP) by islets, with higher concentrations of CRAMP associated with protection from T1D development[167]. CRAMP expression was shown to alter the islet microenvironment, by inducing tolerogenic islet macrophages, regulatory DCs and Tregs, all of which facilitate the reduction of autoreactive T cell activation and thus prevent diabetes development. Altering the microbiota composition by fecal transfer or by antibiotic treatment can change the availability of metabolites, which in turn, alters the risk of developing T1D[168, 169]. Therefore, these options may prove useful in developing prevention therapies. However, the relationship between microbial metabolism and human T1D is still not well understood, and more studies in this area are needed in the future.

Diet and Type 1 Diabetes

Diet is well known to influence microbial composition and functions. In Burkina Faso, Africa, where diets consist of an abundance of complex carbohydrates resulting in higher microbial diversity, children produce greater amounts of SCFAs when compared to children from Europe[170]. In contrast, GF mice colonized with human stool bacteria from an individual with a Western-style diet had less microbial diversity and a worsened ability to metabolize complex carbohydrates. However, those mice given the stool bacteria from the individuals with an enriched microbiota-accessible carbohydrate diet

had increased microbial diversity and were able to metabolize complex carbohydrates[19, 171]. Interestingly, numerous studies have suggested that *Prevotella:Bacteroides* ratios are related to the dietary intake of either complex carbohydrate diets or proteins and fats respectively[170, 172]. Of note, when dietary fibers are almost completely fermented, the pH of the large intestine increases, providing a reduction in butyrate-producing microbiota but an increase in acetate- and proprionate-producing *Bacteroides* bacteria[173].

A barley kernel-based bread diet was introduced to healthy subjects for 3 days in a recent dietary intervention study[174]. The authors identified the improvement of glucose metabolism to be associated with an increased abundance of *Prevotella copri*[174]. The presence of *Prevotella* in these individuals had enhanced enzymatic activity related to breaking down complex carbohydrates, which promotes the generation of a number of SCFAs. The colonization of GF mice with microbiota from the study participants also confirmed an increased abundance of Prevotella that was associated with improved glucose metabolism. Furthermore, branched-chain fatty acid produced by the fermentation of branched-chain amino acids correlated with insulin resistance in germ-free mice receiving stool bacteria from obese human individuals[175].

Dietary influence on T1D has been examined both as a causative agent as well as a preventative or modulating factor. Oral administration of nicotinamide, a vitamin B group substance, to NOD mice prevented the development of diabetes[176]. Further, NOD mice were completely protected from T1D development when administered nicotinamide in combination with a diet consisting of an infant formula, where soy was the source of protein[177]. Vitamin D, specifically the active form, 1,25 di-hyroxyvitamin D, has also been shown to prevent from severe insulitis due to the increased Treg cell activity[178-180].

Gluten, a component of wheat protein, has many antigenic properties, and has been implicated in the pathogenesis of several autoimmune disease states (predominantly CD) but it has also been implicated in T1D. It has been reported that CD can affect up to ~10% of individuals with T1D due to the overlap in *HLA-DR3/DQ2* genetic susceptibility between the two diseases[181, 182]. Furthermore, tissue transglutaminase antibodies are present in some T1D patients, who share susceptibility SNPs to CD in the *CTLA4* gene[183]. Even in those individuals with T1D who were negative for the tissue transglutaminase autoantibody in the serum, the antibody was still found in the

jejunum[184]. Interestingly, T1D patients have increased expression of duodenal inflammatory chemokines and cytokines compared to CD patients and healthy controls[32]. This was also associated with altered microbiota in those T1D patients. Gluten-free diets have been shown to protect NOD mice from the development of T1D[185-187]. Two studies have shown that gluten in the diet affected the quantity and composition of the gut microbiota[185, 186]. Funda and colleagues also reported that while a gluten-free diet prevented diabetes in NOD mice, a gluten-enriched diet also had a preventative effect[187]. They hypothesized that gluten itself is not diabetogenic but can have an immunomodulatory effect on a diabetes-susceptible host by altering the gut microbiota which in turn regulates the immune system. Human studies investigating how diet may influence T1D susceptibility are currently underway. The BABYDIET study is a prospective primary prevention trial recruiting children "at risk" of developing diabetes, who have a first-degree relative with T1D and carry a T1D-risk HLA genotype. Children were randomly assigned to one of two groups whereby gluten was introduced in the diet at 6 months or 1 year of age[188]. Data from the participants within the first 3 years of age showed similar prevalence of islet autoimmunity regardless of when gluten was introduced; however, these children will continue to be followed over time, to observe generation of autoantibodies and other markers that are associated with the development of both celiac and T1D autoimmunity.

Antibiotic usage and Type 1 Diabetes

There are many different classes of antibiotics that target different bacterial pathogens. In addition to clearing the pathogen(s) from the original infection in various body sites, antibiotics can alter the gut microbiota directly, particularly as most of the antibiotics are taken via oral route. Will antibiotic usage affect T1D development by changing gut microbiota? Studies in mouse models of T1D suggest that the type of antibiotics, time of usage (early in life or later in life) and the duration of usage can modify the susceptibility of T1D development. Table 1 summarizes different studies conducted to assess the role of microbial modulation in the development of T1D in NOD

mice. We have shown that vancomycin, which predominantly targets Gram-positive bacteria, promoted T1D development in NOD mice, whereas neomycin, which targets Gram-negative bacteria, protected NOD mice from T1D development[189, 190]. Interestingly, others have reported that life-long vancomycin usage protected NOD mice from T1D development[191]. It is clear that not all antibiotics have the same impact on T1D development. Some antibiotics can protect NOD mice from the development of T1D[189-191], while others promote the development of diabetes in NOD mice[168, 192, 193]. Interestingly, we found that the treatment of combination of four different antibiotics (ampicillin, vancomycin, metronidazole and neomycin, AVMN) had no effect on diabetes development in NOD mice despite the fact that the AVMN treatment depleted most, if not all, gut bacteria (Peng, *et al.*, unpublished). This highlights the observation that the treatment protocol can also influence diabetes development. In the study showing that vancomycin protected NOD mice, the mice were treated from birth to 28 days of age or from 8 weeks of age to termination[191]. However, in the studies showing that vancomycin accelerated T1D development, NOD mice were treated from conception to termination or from conception to 24 hours post-birth[190, 193]. The different treatment protocols, including dose, duration and starting age for these antibiotic studies make it difficult to compare the outcome of different studies directly. Moreover, the gender of the mice used in the studies may also affect diabetes development. As discussed earlier, sex hormones influence the gut microbiota and T1D susceptibility in the NOD mouse[138, 153]. While most studies have used female mice, some studies have been conducted in both genders. Candon and co-authors found that long-term (from conception to the progeny at 40 wks of age) treatment with vancomycin or a combination of streptomycin, colistin and ampicillin (Strep-Col-Amp) significantly increased diabetes development in male NOD mice but had no effect on female mice[193]. In another study, male NOD mice receiving tylosin also exhibited increased diabetes development[168]. Interestingly, the female NOD mice, also exhibited increased diabetes development in one animal facility but this was not reproducible in a second animal facility. This raises an important point that the resident gut microbiota and animal facility standards can influence the experimental outcome. This also raises the issue of reproducibility of studies as small microbial changes may influence the effectiveness of the antibiotic treatment. Clearly,

these studies are not possible in humans; however, epidemiological investigations into antibiotic usage and T1D development in humans have not shown any evidence that antibiotics administered to children have influenced the onset of T1D.

Future Directions: Manipulating the gut microbiota as a novel therapy

Probiotics

Probiotics are microorganisms that may have health benefits by modifying the gut microbiota and improve nutrient absorption, enhance immune regulation and protect the host from infection and disease[194]. While many of the probiotics may not colonize their hosts in the long-term, they do have important immunomodulatory effects in the short-term[195].

Probiotics have been shown to protect NOD mice and BB rats from developing T1D[196-199]. *Lactobacillus casei,* which is believed to be a probiotic strain, can protect NOD mice from T1D development when administered in the diet from 4 weeks of age[196]. This protection was associated with decreased splenic CD8 T cell number and increased IL-10 and IL-2 cytokines with age. The probiotic VSL#3 is a mixture of bifidobacteria (*B. longum*, *B. infantis* and *B. breve*), lactobacilli (*L. acidophilus*, *L.casei*, *L. delbrueckii subsp. L. bulgaricus* and *L. plantarum*) and a strain of streptococcus bacteria (*Streptococcus salivarius subsp. thermophilus*)[197]. In a study, Calcinaro and colleagues administered VSL#3 to NOD mice, three times per week from 4 to 32 weeks of age and this protocol led to significant protection from T1D development in treated NOD mice compared to non-treated controls (21% vs 81% respectively). VSL#3 administration decreased expression of IL-1β and increased expression of indoleamine 2,3-dioxygenase and IL-33, both of which have tolerogenic properties[199]. However, in another study, VSL#3 was administered via the drinking water to pregnant mice, just before birth until termination of the progeny (22 wks old), and this did not protect NOD mice from the development of T1D[169]. In a very recent study, Hanninen and colleagues reported that *Akkermansia muciniphila* abundancy is negatively correlated to T1D development and oral transfer of *A. muciniphila* delays diabetes development in NOD/Jax mice that, otherwise, have an early disease onset[200]. However, *A. muciniphila* did not reduce the

overall incidence of diabetes[200], whereas gavage of *Clostridium butyricum CGMCC0313.1* was able to significantly reduce the incidence of diabetes in NOD mice by promoting regulatory T cells[201].

There have been some studies investigating probiotics and host immunity in humans. Treating CD children with two *Bifidobacterium breve* strains, Primec and co-authors identified changes in microbiota composition that were associated with the changes in SCFAs and reduced TNF α in circulation[202]. The authors also found that *Verrucomicrobia, Parcibacteria* and some other bacteria yet unknown phyla were strongly correlated to TNFα[202]. The probiotic administration to the CD children lowered the level of TNFα and reduced the abundance of *Verrucomicrobia*[202]. This may provide potential therapeutic targets for CD. However, in a different disease setting, a reduced proportion of *Verrucomicrobia* has also been associated with glucose intolerance in type 2 diabetes patients and prediabetes subjects[203]. There have also been a few studies in T1D. A double-blind randomized pilot study investigated how *Lactobacillus johnsonii* N6.2, which was shown to delay T1D onset in BB rats[198], affects the host immunity in healthy adults[204]. In this phase I human study, only healthy individuals without gastrointestinal disorders or other health issues (e.g. diabetes, mental diseases, kidney and heart diseases and others) were recruited for the study. The administration of *L. johnsonii* N6.2 (taken in capsules for over 8 weeks) resulted in an increase of tryptophan in the circulation[204]. It is known that tryptophan promotes Treg cell induction and expansion as well as suppresses the differentiation of Th1 cells. These data suggest that *L. johnsonii* N6.2 is safe for treatment in adults, although the authors found that the study subjects had increased monocytes, NK cells and CD8 T cells in the peripheral blood compared to the placebo controls[204]. It is clear that more studies need to be done before launching clinical trials in the subjects with T1D or those at risk of developing T1D.

The Environmental Determinants of Diabetes in the Young (TEDDY) study follows children at risk of developing T1D to understand how different environmental factors may influence T1D susceptibility in humans. Recent data from the TEDDY study have revealed a large variability in the fecal microbial probiotic (total lactobacilli and *L. plantarum*) compositions between the children, particularly before 10 months of age[205]. It is currently unknown whether those with a reduced abundance of lactobacilli in early

life will have a greater risk of developing T1D; however, the TEDDY study shows the presence of probiotic-related bacteria in children who are at risk of developing T1D, and correlating this with development of diabetes, over time, will be of considerable interest. This is an ever-expanding area with more probiotic-based human T1D clinical trials planned.

Fecal Microbiota Transplantation

Fecal microbiota transplantation (FMT) can change the gut microbiota in the recipients to elicit health benefits. FMT involves processing stool from a healthy donor (for allogeneic transplants) or from the recipient (autologous transplant), although more often the FMT is an allogeneic transplant from a healthy donor. Sometimes the FMT recipients may need to be treated with antibiotics 24-72 hours prior to FMT to eliminate any existing deleterious gut microbiota. An alternative approach to fast, and have bowel preparaton shortly before the treatment. FMT has been used for the treatment of severe *Clostridium difficile*-induced colitis[206]. Many studies confirmed that FMT therapy is safe and effective in treating human IBD[207]. Interestingly, FMT (from lean donor feces) improves insulin sensitivity in individuals with metabolic syndrome[208].

In T1D, promising results using FMT have been shown in mice. Our study showed that fecal materials from *MyD88*$^{-/-}$NOD mice, which were protected from diabetes, can delay and reduce diabetes development in NOD mouse recipients [209] However, there has been no report to date on whether FMT could be beneficial in ameliorating disease in T1D patients or in preventing T1D development in the individuals who are at risk of developing T1D. Both NOD mouse studies and recent TEDDY studies have pointed to early life as an important window of opportunity for effective intervention to reduce islet autoimmunity, especially in relation to the composition of gut microbiota[56] and possibly FMT.

Concluding remarks

Increasing evidence suggests that there are microbial perturbations in individuals with islet autoimmunity or T1D compared to healthy control subjects; however, we are

still not clear about the defined mechanisms. Therefore, further functional studies are needed, not only to probe the interaction of microbiota with immune system but also to identify the causal link(s) between the presence of certain gut bacteria and diabetogenic autoimmune responses. Germ-free NOD mice will provide a valuable tool in deciphering the role of the microbiota in relation to the development of T1D, both *in vitro,* and more importantly, *in vivo*. While GF mice are known to have a less mature immune system, particularly in relation to the gut-associated mucosal lymphoid tissue[210], GF NOD mice, still develop T cell-mediated T1D[71, 138, 153, 211]. Thus, using these GF NOD mice as recipients for human stool from diabetic donors and healthy control donors, allows us to understand how the microbiota may modulate the immune system in T1D *in vivo*. Studies in obesity and T2D have shown that human gut microbiota can improve glucose control and insulin resistance and thus manipulation of the microbiota may also be used to achieve better glycemic control in patients with T1D.

Diet and probiotics may provide easier acceptance and compliance in participants for disease prevention and/or intervention and/or modulation of disease. However, there are still some challenges e.g. in dietary interventions, the subjects have to be at the age of taking solid food. As for the use of probiotics, the question remains as to how long the beneficial immunomodulatory effects induced by probiotic will last and if further doses are required. Moreover, while FMT may alter the gut microbiota in the longer term, many of these studies pre-treat with antibiotics which provides a niche allowing newly introduced microbiota to colonize. It is noteworthy that antibiotic treatment may lead to antibiotic resistance. In addition, it is highly possible that FMT-induced microbiota changes can be modified by diet and other factors. Therefore, for future therapy development, we require further understanding of 1) microbial community interactions as a complex ecosystem; 2) the interaction of host microbiome with intestinal micro-environment including gut epithelial cells and specialized gut endocrine cells including Goblet cells and Paneth cells and 3) the interaction of gut microbiota with the host immune cells locally and systemically. Furthermore, we also need to better define subgroups of patients, as increasing evidence suggests that T1D is not a homogenous disease condition. If we can combine the information on microbiota with clinical data, to truly decipher the results by subgrouping patients based on resident microbiota

prevalence, c-peptide concentrations, autoantibody presence and type, as well as autoreactive T cell information, we will be able to design treatment with more precision. In summary, the role of microbiota in T1D is complex. While there are associations with microbial composition changes at the time of seroconversion and changes associated with disease onset, it remains unclear if the microbiota play a causal role in human T1D development. However, studies in NOD mice have shown that microbial antigens stimulated diabetogenic CD8 T cells and accelerated T1D development[103, 212]. It is clear from mouse and human studies, the microbiota can induce both proinflammatory and anti-inflammatory changes of the immune system.

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