Antibiotics, gut microbiota, environment in early life and type 1 diabetes

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Abstract

The gut microbiota interact with innate immune cells and play an important role in shaping the immune system. Many factors may influence the composition of the microbiota such as mode of birth, diet, infections and medication including antibiotics. In diseases with a multifactorial etiology, like type 1 diabetes, manipulation and alterations of the microbiota in animal models has been shown to influence the incidence and onset of disease. The microbiota are an important part of the internal environment and understanding how these bacteria interact with the innate immune cells to generate immune tolerance may open up opportunities for development of new therapeutic strategies. In this review, we discuss recent findings in relation to the microbiota, particularly in the context of type 1 diabetes.

1. Gut microbiota and type 1 diabetes

Type 1 diabetes (T1D) is a T cell-mediated autoimmune metabolic disease which is commonly seen in children and young adults (1) although it can also present in older adults. The insulin-producing beta cells of the pancreatic islets are damaged and destroyed by activated autoreactive T cells resulting in disordered blood glucose regulation (2). This destruction is the result of a complex interaction between genetic susceptibility genes and environmental factors (3, 4). Genetic screening has shown that certain major histocompatibility complex (MHC) class II genes, also called human leukocyte antigen (HLA) genes, *DQA1*0301* (DQ2), *DQB1*0302 (*DQ8), *DRB1*DR301* (DR3) and a number of DR4 alleles are associated with susceptibility to T1D in patients (5, 6). However, only a small portion of individuals carrying those alleles will develop T1D (7). Yet, a sharp rise of T1D incidence has been seen in recent years (8) in a time frame that is not sufficient for genetic change, indicating that environmental factors may play a crucial role in diabetes development (9). Prenatal influence, viral infections, dietary factors in the young as well as “hygiene” can all affect the disease onset (10). More recently, several studies have shown commensal microbiota to be connected with the development of this autoimmune disease (11). Although triggering factors for T1D have not yet been clearly identified, the gut microbiota are believed to play an important role in the development of the disease (12, 13).

The gut microbiota are associated with the development of several diseases including obesity and type 2 diabetes (14), liver disorders (15), intestinal inflammatory syndromes

(16), allergic diseases (17), disorders in the central nervous system (18), and especially, autoimmune diseases (19-22). We, and others, have recently reported that alteration of gut microbiota by pharmacological means can protect from or accelerate T1D development in non-obese diabetic (NOD) mice (23-27), a well established animal model for T1D research (28).

We were among the first to demonstrate that the gut microbiota shape the NOD mouse innate immune system (11). MyD88 is a central adaptor in most innate immune Toll-like receptor signaling pathways and MyD88-deficient NOD mice do not develop autoimmune diabetes in a clean, but not sterile, housing environment; however, germ-free MyD88-deficient mice develop full-blown diabetes (11). This indicates that commensal bacteria, especially gut bacteria play a very important role in triggering the autoimmune disease. When a defined microbial mixture was introduced orally into the germ-free MyD88-deficient mice, diabetes development in these mice was attenuated (11). Similar results were later observed in different mouse models of human diseases including Celiac Disease (29), obesity/type 2 diabetes (30), and autoimmune uveitis (31).

There are 10-fold more microorganisms residing in the gut than the total number of human cells (32), and they protect the host from infection by various pathogens (33). The main roles of gut bacteria are to aid in nutrition derived from the diet and to generate energy. A healthy microbiota composition helps to keep the gut epithelia intact and reduce permeability (34, 35). Furthermore, the interaction between gut

epithelia and the bacteria promotes the development of a normal immune system (36, 37). Several reports have demonstrated that colonization by some specific bacteria in the gut can protect mice from developing type 1 diabetes; these bacteria include SFB (Segmented Filamentous Bacteria) (38), *Lactobacillus johnsonii* N6.2 (39), as well as some Streptococcal species (40), and glycoprotein extracts from Klebsiella pneumoniae (41).

2. Modification of the gut microbiota

Although controversial, germ-free mice (11, 42-44) may have accelerated T1D. Conversely, there has been speculation that gut bacteria may trigger T1D development in genetically susceptible humans (45) and mouse models of T1D (46). One possible means by which this could occur could be transfer of metabolites or cell components of the bacteria through a "leaky" gut wall and uptake by antigen presenting cells, processing and presentation of the antigen to activate T cells (47). Tight junctions represent the major barrier within the paracellular pathway between intestinal epithelial cells. Alterations in intestinal permeability allow access of bacterial toxin (45), infectious agents and dietary antigens from the lumen to mucosal immune elements (48, 49). Another possible mechanism is some bacterial product(s) share the molecular homology with islet autoantigen(s) and the islet beta cells are attacked by the immune cells that are reactive to the bactierial antigens (46).

Autoantibodies have been observed in T1D patients as young as several months old (50,

51). Animal model studies have also shown that alteration of gut microbiota early in life, and gut permeability are important in shaping the host immune system (25, 52, 53), especially at the prenatal or neonatal stages.

Efforts have been made to investigate which bacteria in the gut may be beneficial or harmful in the development of T1D (45, 46, 54-57). Researchers have studied altered gut microbiota in experimental mice after treating with a combination of 4 antibiotics, including Ampicillin, Metronidazole, Neomycin and Vancomycin (58). Although there are studies using germ-free (GF) mice to test whether one or more species of bacteria introduced into the mice has an impact on diabetes development (42, 59), which species are probiotic and which are detrimental have not been conclusively determined, as most of the bacteria in the gut are non-culturable.

Other studies have been conducted using vancomycin, a specific gram-positive bacterial inhibitor, to modify the gut bacteria. Antibiotic intervention during the prenatal period revealed an acceleration of diabetes onset (27, 60), whereas NOD mice receiving vancomycin from birth onwards gave the opposite result (52). Recently, Brown and colleagues showed that using Neomycin and Vancomycin to treat NOD mouse pups from the neonatal period for their lifetime (61) accelerated diabetes development. These studies indicated that the time at which antibiotic treatment is commenced is crucial and that treating the mothers may be a way of having an effect while avoiding direct administration of the antibiotics to the pups. Many of these studies used an approach giving long-term antibiotic treatment, although long-term antibiotic treatment rarely

occurs in humans. Thus, the advantage of studying short-term treatment makes the studies in animals closer to humans (25, 27). In addition, human studies have shown that approximately 30% of pregnant women in the USA have had a short-course of antibiotic medication during their pregnancy (62) and the number could be higher in other countries. It should be noted that long-term antibiotic treatment could cause resistant bacteria to propagate in the gut (63).

3. Protective bacteria that arise from pharmacological alteration of gut microbiota in early life.

The colonization of gut microbiota is strongly influenced by microbial exposure at birth (64). When antibiotic treatment is used to study the effect of gut microbiota on disease, it is clear that the timing of administration, duration of treatment, as well as the type of antibiotic used must be taken into account. We have published a study showing that Neomycin/Polymyxin B/Streptomycin- treated NOD mice were protected from T1D development (25). This protection was more significant when mice were treated at the prenatal stage (Figure 1, adapted from reference (25))

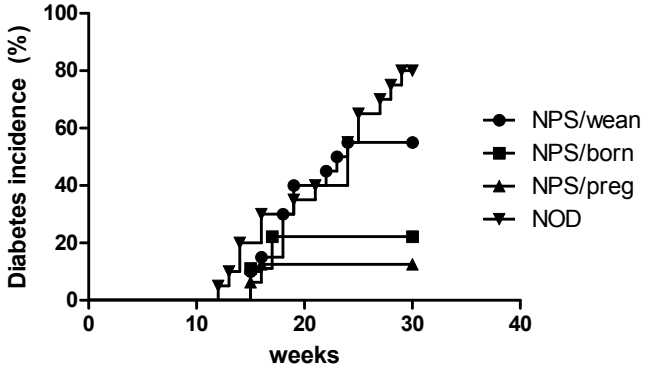

Figure 1: Maternal antibiotic treatment protects offspring from diabetes development in NOD mice. Antibiotic treatment (3 week) starting at different times of life led to a different phenotype of T1D development. NPS (Neomycin/Polymyxin B/Streptomycin); NPS/preg (NOD offspring from mothers treated with NPS during pregnancy); NPS/born (NOD treated with NPS from birth to weaning); NPS/wean (NOD treated with NPS immediately after weaning). The change in diabetes incidence was dependent on the time of antibiotic treatment.

It is clear that the earlier the NOD mice received the antibiotics, the better the protection from diabetes development. In our study, NOD mice treated with NPS at different time points early in life delayed and overall reduced T1D onset. When pregnant mothers were treated with antibiotics, the offspring were most protected from diabetes, while mice receiving antibiotics from birth or weaning were also protected from disease development although this was not statistically significant. Here, not only did the NPS treatment generate a gut bacterial composition that was protective but it was also clear that the timing of treatment was very important in

inducing the effects. Overall, these studies suggest that gut microbiota in very early life (prenatal or neonatal) may have the most positive impact on the host immune system. Other studies have demonstrated that microbial exposure during early life is important for development and maintenance of the immune system and germ-free mice are more susceptible to developing T1D (42, 43).

Considering which bacteria have protective effects in relation to diabetes development, we have observed that the Gram-positive Firmucutes *Lachnospiraceae* and *Coriobacteriaceae* significantly increased in prenatally NPS treated mice (25). Several studies in mice and human case reports indicated that, in both BioBreeding Diabetes-Prone rats (65-67) and diabetic children (68, 69), decreased *Bacteroidetes*, together with an increase in other Gram-positive Firmicutes such as *Lactobacillus*, *Bifidobacterium*, were found, compared to BioBreeding Diabetes-Resistant rats and healthy children, respectively.

SFB (Segmented Filamentous Bacteria) are a group of bacteria within the Genus *Candidatus Arthromitus* which belongs to the Phylum of Firmicutes, Class of *Clostridia* and Family of *Lachnospiraceae*(70). SFB were found to induce intestinal Th17 cells in Lamina Propria (LP) (71) and there are reports showing that colonization of the gut of NOD mice with SFB can induce a substantial population of Th17 cells in the LP and protect the female NOD mice from diabetes development (38). However, we did not find that SFB conferred diabetes protection in NOD mice (27). A study by Yurkovetskiy and colleagues also showed that SFB did not protect GF NOD mice from diabetes

development; however, SFB reduced T1D development in male GF NOD mice after colonization with other gut bacteria (72). Since SFB are a group of bacteria, genomic sequencing results from several SFB strains have shown that they are different from each other (73-76). It is, thereofre, conceivable that different strains may have different biological effects. Nevertheless, it is still important to study the role of SFB in mice because these gram-positive, anaerobic, commensal bacteria are capable of inducing the postnatal maturation of homeostatic innate and adaptive immune responses in the gut. A recent publication also showed that not only can colonization with SFB induce IL-17A but CXCR2-dependent recruitment of neutrophils in the gut also occurs (77).

4. Shaping the immune system via alterations in gut bacteria

How does alteration of the gut bacteria lead to protection from diseases like type 1 diabetes? There are a number of proposed mechanisms which are associated with genetics, gut microbiota (related to antibiotic usage, mode of delivery, diet) and infection in animal models and humans (78, 79). Our recent studies indicate that protection can be mediated by tolerogenic antigne presenting cells (APCs) originating in the gut associated lymphoid tissue (GALT), which have reduced ability to stimulate cytotoxic CD8+ T cells. (Figure 2, Cover figure)

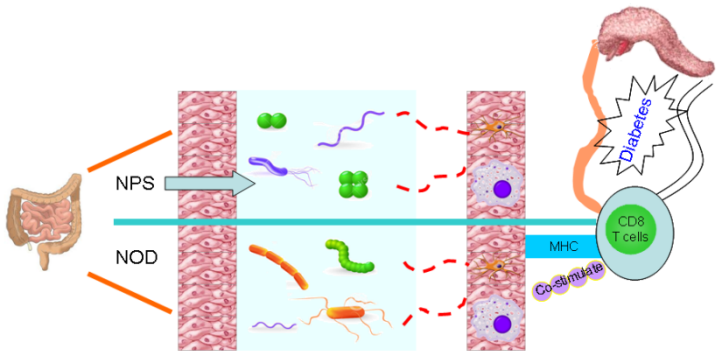

Figure 2: How do antibiotics affect pancreatic beta cell autoimmunity: gut microbiota composition is altered after antibiotic (NPS) treatment and antigen presenting cells exposed to the altered bacteria in the gut display impaired antigen presenting ability to CD8 T cells, which in turn alleviate insulitis in the pancreas and protect the host from diabetes development.

In supporting the mechanism of tolerogenic APCs as a result of altered gut microbiota due to antibiotic usage, Umenai and coauthors showed hyporesponsiveness of macrophages, a potent subset of APCs, in response to LPS stimulation in mice after Streptomycin treatment (80). Similarly, a recent study demonstrated that dendritic cells became hyporesponsive to LPS stimulation and reduced inflammatory cytokine production upon exposure to the gut bacterium *Lactobacillus reuteri* (81). Dolpady and co-authors reported that administration of a mixture of *Bifidobacteriaceae*, *Lactobacillaceae* and *Streptococcus* in 4-wk old mice promoted tolerogenic $CD103^{+}$ dendritic cells and reduced Th1 and Th17 cells in mucosal and PLN sites (82). Thus, alteration of gut microbiota by different means including antibiotic treatment can

affect APCs lead to acceleration of or protection from diabetes development. The changes in APC function could be the result of direct contact between APCs and gut microbiota in the gut or indirect contact, mediated by metabolites from altered gut microbiota. Due to the immature nature of gut barrier and the changing, maturing community of gut microbiota in early-life, timing becomes critical.

Reduction in regulatory T cell markers have also been postulated as a mechanism for protection of NOD mice after treating with a mixture of antibiotics – metronidazole, streptomycin and polymyxin prenatally (53, 60). These studies demonstrated that the changes in the immune system occurred when antibiotic treatment was given prenatally, a particularly important time for immune system development. Livanos and coauthors reported recently that NOD mice receiving antibiotics (penicillin V) from lactation until the age of 40 days had earlier diabetes onset and overall higher incidence of diabetes (83). This early-life treatment reduced the percentage of Treg and Th17 cells in lamina propria (LP), which may have contributed to the accelerated diabetes development. In the Streptozotocin (STZ)-induced type 1 diabetes model, mice treated with antibiotics were fully protected from diabetes (84). This was attributed to blocking pro-diabetic bacteria translocation to PLN (84).

Early-life treatment using vancomycin reduced the incidence of diabetes in one study (52) but accelerated diabetes in another (27). The discrepancy may be attributed to

different treatment protocols. However, the protection in Hansen's study was accompanied by an increase in the level of *Akkermansia*, which was later reported to be correlated with a pro-diabetic effect (85, 86). A gluten-free diet can also reduce inflammation and diabetes incidence in NOD, with elevated abundance of *Akkermansia*. Adding gluten to the gluten-free diet reversed the protection, accompanied by a decreased level of *Akkermansia* (86). More recently, a study showed that *Akkermansia* mediate glucose tolerance via IFNγ (87) using loss-and-gain-of-*Akkermansia muciniphila* approaches in IFNγ knock-out mice. In a clinical study, a 4-day treatment with broad-spectrum antibiotics (Vancomycin, Gentamicin and Meropenem) significantly shifted the gut microbiota composition but this did not have a clinical impact in respect of metabolic markers such as glucose tolerance or insulin secretion (88). However, in a study by Endesfelder and coauthors a change of gut microbiota occurring as a result of dietary change had an impact on islet autoimmunity. The authors stratified the children in the study based on the microbial communities identified. They found that it was possible to detect functional associations between the diet consumed, the microbiome and development of autoimmunity. They identified a subgroup of children where *Bacteroides* was dominant, with low *Akkermansia* in the gut microbiota and this was associated with early introduction of a non-milk diet, lower abundance of genes for the production of butyrate and early autoantibody development (89, 90). They postulated that low butyrate generation by the bacteria contributed to increased risk of the development of islet autoantibodies (90).

It is clear that the effect of gut microbiota on host immune responses early in life is much stronger than later in life. This was observed in human studies demonstrating that the development of type 1 diabetes was closely related to gut microbiota, gut permeability and immune system in early-life. Amarri and colleagues have previously found that gut bacteria and gut permeability as well as other immune markers were significantly altered in breast-fed infants (89). Other early influences have been demonstrated in a Danish study where the authors found that antibiotics used in early-life increased the incidence of type 1 diabetes in young children: however, this was also related to the mode of birth delivery, which has major effects on determining the composition of gut microbiota at the time of birth (91).

Antibiotic treatment in adult mice is not as effective as prenatal or neonatal treatment in altering the course of type 1 diabetes. However, later life antibiotic treatment can significantly alter the gut bacteria and it can have clear impact on diseases other than T1D, including Crohn's disease (92), colitis (93), obesity and type 2 diabetes (94).

In addition to antibiotics, it is known that innate immune system can also alter gut microbiota, which affect both type 1 and type 2 diabetes development (11, 30). However, it was not clear which type of bacteria contribute to the protection or promotion of diabetes. Using a MyD88-deficient NOD mouse that has a defined T cell receptor reperotire, we recently found that one type of bacterium, *Leptotrichia*

goodfellowii, in the gut can trigger diabetes development in islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-reactive CD8 T cell receptor NY8.3 transgenic NOD model. *L. goodfellowii* is a member of *Fusobacteria*, and expresses a protein peptide sharing a homologous sequence with IGRP and its abundance is correlated with the progression of diabetes (46). This is the first evidence of molecular mimicry that relates to a bacterial antigen in initiation of diabetes onset, due to T cell cross-reactivity to the microbial peptide and leading to an activated autoimmune response.

5. Fecal microbiota transfer (FMT) as a potential therapy

Gut bacterial composition may be altered by diet, antibiotics, probiotics, or by direct microbiota transfer. Fecal microbiota transfer (FMT) has proven to be a effective way to transfer "healthy" microbiota, that may have beneficial effects on metabolism and the immune system in recipient mice (30, 95). FMT has also been used in patients with colitis (96-98) and recently in cancer patients (99, 100). We recently demonstrated that FMT could restore the gut microbiota and rebalance the gut hemeostasis, which in turns delayed diabetes onset in NOD mice (26, 29). In other disease models, including obesity, the gut microbiota have been shown to contribute to generation of the metabolic syndrome (30). FMT from lean mice to obese mice can ameliorate the metabolic syndrome in the obese mice due to the rebalancing of gut microbiota (101). Treating metabolic syndrome with FMT has also been tested in humans [98].

The beneficial effects of FMT have been particularly used in patients suffering from *Clostridium difficile* infection (102, 103). Although antibiotic treatment has been the mainstay of treatment of *Clostridium difficile* infection, some patients have developed recurrent infection later on and importantly antibiotic treatment for other medical conditions can lead to persistent infection with *Clostridium difficile* (104-106). Since the clinical trial on FMT in patients with recurrent *Clostridium difficile* infection (103), considerable progress has been made in this field, including formulation of the microbiota and delivery route of the bacteria. Oral administration is very common using capsules containing the gut microbiota of healthy donors after pathogen-free screening, stool purification and preservation processes (107). In addition, FMT has also been effective in treating diseases like ulcerative colitis (108) and chronic pouchitis (109) in clinical practice. Although FMT has not been used in T1D treatment, if specific combinations of beneficial bacteria could be identified, this could be a promising therapy, given that fecal filtrate has a similar efficacy to fecal microbiota in treating other medical conditions (110). However, more comprehensive studies need to be performed to understand which what bacterium (or bacteria) and metabolites can trigger the imbalance of immune system at early-life in humans at high-risk of developing T1D.

6. Perspectives and conclusion

It is clear that many factors can affect the composition of gut microbiota, which

modulate the immune system contributing to health or disease including T1D. Birth delivery mode (cesarean section vs. vaginal delivery), breast vs. formulation feeding and antibiotic usage, all can have a strong impact on the health of newborn infants (111-113). The period in utero, as well as post-partum, is a crucial time for establishment of the immune system of the babies. Intervention during early life may lead to novel and more effective therapeutic strategies in treating T1D. There is no doubt that gut microbiota are associated with particular metabolites and can influence both metabolic pathways and the development of the immune system. Many studies have shown that depletion or alteration of intestinal microbiota has a significant impact on gut mucosal and epithelial gene expression, as well as immune responses (114). Gram-negative and Gram-positive bacteria can stimulate different immune responses and induce Th1 or Th2 type cytokines, as well as proinflammatory or anti-inflammatory mediators (115-118). We summarized some of the bacteria studied in association with T1D (Table 1).

APC dysfunction has been found to be associated with T1D development (119, 120), but previously this had not been correlated with alteration of gut bacteria. Our recent studies provide evidence that tolerogenic APC are generated as the results of altered gut microbiota (25, 27). Interestingly, the tolerogenic APCs can confer T1D protection and this protection can also be transferred to a second host and to the offspring. Probiotics and other strategies altering the gut microbiota, including FMT, could be a promising approach to modulate gut microbiota and rebalance the homeostasis of

mucosal and systemic immune systems. This “bug for drug” approach, also called “Bacteriotherapy” is being tested in different clinical trials for other medical conditions (121). The prospect of this approach for T1D could be on the horizon. Type 1 diabetes has not only a genetically inherited component that determines susceptibility to disease, but the interaction with the environment to precipitate disease is a very important part of the pathogenesis. Identifying modifiable environmental factors, such as the gut microbiota would provide a therapeutic target that could be modified by treatments that could be easy to administer. It would be important to identify safe means of doing this that could potentially be administered very early in life. Probiotics have already been tested in infants and young children but the appropriate composition should be identified. Whether gut bacteria are involved in the initiation of the process leading to T1D or in the progression of β-cell autoimmunity is still unclear (122). A pure, culturable bacterial cocktail would need to be identified before this kind of regimen could be used in clinical practice.

A number of immunotherapeutic treatments have been trialed in human type 1 diabetes, some of which have had a transient effect, but none as yet have been long-lasting (123). It has been argued that for a treatment to be successful, the innate immune system should present antigens in a tolerogenic manner to T regulatory cells (123) and this is potentially one of the ways in which altering the gut microbiota could work if the right combination could be identified. Ideally, this type of treatment would

be combined with others that could be synergistic in maintaining a tolerogenic environment.

In conclusion, both probiotic and antibiotic treatment can significantly alter the gut bacteria, as well as the bacteria composition in other sites of the body including the oral cavity. The altered bacteria interact with the host immune system and reduce inflammatory cytokines as well as the expression of costimlatory molecules in APCs, inducing a tolerogenic environment. However, further investigation is required and, in particular, more work done to identify less disease- promoting gut microbiota and how to maintain this type of gut microbiome.

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Table 1: Summary of bacteria studied in association with T1D

Bacteria	**Gram-positive or -negative**	**Mode of action**	**Protection from or acceleration of T1D**	**References**
SFB (Segmented filamentous bacteria), *Candidatus Arthromitus*	positive	Induce Th17 response	Protection No effect	38 27
Lactobacillus johnsonii & L. reuteri	positive	Healthy probiotics	Protection	39, 81
Klebsiella pneumoniae	negative	Pathogen causing pneumonia	Protection	41
Bifidobacterium	positive	Often as healthy probiotics	Acceleration	67-69
Streptococcus	positive	Some pathogens, some commensal bacteria in mouth, skin, intestine and upper respiratory tract	Protection	40, 82
Akkermansia muciniphila	negative	Mucin-degrading, often anti-inflammatory effect	Protection	52, 90
Bacteroides	negative	processing complex molecules to simpler compounds in the host intestine	Acceleration	89, 90
Leptotrichia goodfellowii	negative	Oral commensal, pathogen in immune compromised patients	Acceleration in NY8.3 NOD	46