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Type 1 diabetes: role of intestinal microbiome in humans and mice

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Type 1 diabetes is a disease involving autoimmune destruction of pancreatic beta cells in genetically predisposed individuals. Identifying factors that trigger initiation and progression of autoimmunity may provide opportunities for directed prophylactic and therapeutic measures to prevent and/or treat type 1 diabetes. The human intestinal microbiome is a complex, symbiotic ecological community that influences human health and development, including the development and maintenance of the human immune system. The role of the intestinal microbiome in autoimmunity has garnered significant attention, and evidence suggests a particular role for intestinal microbiome alterations in autoimmune disease development, including type 1 diabetes. This review will examine the role of the intestinal microbiome in the development and function of the immune system and how this relates to the development of autoimmunity. Data from animal and human studies linking alterations in the intestinal microbiome and intestinal integrity with type 1 diabetes will be closely examined. Finally, we will examine the interactions between the intestinal microbiome and dietary exposures and how these interactions may further influence autoimmunity and type 1 diabetes development.

Keywords: type 1 diabetes; intestine, microbiome; gliadin

Introduction

Type 1 diabetes is an autoimmune disease resulting in the destruction of insulin-secreting beta cells of the pancreas. The resultant lack of insulin results in hyperglycemia that is secondarily associated with micro- and macrovascular complications. Lifelong exogenous insulin replacement remains the mainstay of therapy for the majority of patients with type 1 diabetes. Despite improvements in insulin therapy, glucose monitoring, and glycemic control, life expectancy is shorter, and quality of life is significantly compromised, compared to the general population.[1–3] The economic burden is also significant. Each year in the United States, type 1 diabetes accounts for 14.4 billion dollars in medical costs and lost income.[4] These factors have prompted substantial research efforts to define the pathophysiology of type 1 diabetes in order to develop more efficacious therapies or to prevent the disease all together. Recently, the intestinal microbiome, encompassing the entire bacterial community of the intestine, has garnered significant attention for its role in normal health and development and potential role in disease including type 1 diabetes. The following will review type 1 diabetes pathogenesis, the study of the intestinal microbiome, and the potential role that alterations in the microbiome and intestinal integrity may have in the development of type 1 diabetes.

Type 1 diabetes mellitus

Incidence

The incidence of type 1 diabetes has increased dramatically in developed countries over the past several decades. The majority of data collected regarding type 1 diabetes incidence comes from large registries, including a European registry, EURODIAB (Epidemiology and Prevention of Diabetes), and the DiaMond network, encompassing 57 countries, including the United States, China, and several European countries. The EURODIAB registries

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revealed a 3.9% annual increase in the incidence of type 1 diabetes from 1989 to 2003.[5] Similarly, DiaMond revealed a 2.4% annual increase in incidence between 1990 and 1994 and 3.4% between 1995 and 1999.[6] In the United States specifically, data previously revealed a steady or modestly increasing incidence of type 1 diabetes.[7] Recent data from the SEARCH for Diabetes in Youth study, however, suggest that the incidence of type 1 diabetes in the United States is higher than previously thought with 24.3 cases per 100,000 person-years, rivaling the incidence seen in other large, multinational registries.[8] The rapid increase in the incidence of type 1 diabetes in developed countries suggests that nongenetic factors contribute to the pathogenesis of the disease. Specifically, environmental factors including dietary changes, alterations in infectious disease exposures, and increased pharmaceutical use, especially antibiotics, may contribute to the development of the disease.

Pathophysiology

The pathophysiology of type 1 diabetes is complex and still not entirely understood. The clinical manifestations of type 1 diabetes represent the end stage of several distinct pathogenic processes. Genetic predisposition combined with environmental factors initiate the process of autoimmune destruction of the beta cells of the pancreas, a process that involves both the innate and adaptive immune systems. The beta cell destruction remains subclinical until approximately 80% of the beta cell mass is destroyed, at which time hyperglycemia ensues.[9] Eventually, near complete or complete loss of beta cells occurs resulting in significant insulin deficiency, worsening hyperglycemia, and the absolute necessity of exogenous insulin therapy.

Genetic susceptibility is a key factor in any individual's risk for developing type 1 diabetes. To date, several specific genetic risk factors for type 1 diabetes have been identified; and to further emphasize the role of genetics in the pathogenesis of type 1 diabetes, monozygotic twin siblings of individuals with type 1 diabetes have been shown to have a 50% risk of developing type 1 diabetes.[10] Specific polymorphisms in the major histocompatibility complex (MHC), immune molecules normally present on the surface of antigen presenting cells (APC), were the first genetic factors noted to be associated with an increased likelihood of type 1 diabetes. Specifically, the presence of HLA alleles DQ and DR significantly increases the likelihood of type 1 diabetes, though the amount of risk is in large part determined by environmental factors.[11,12] Underscoring the significance of HLA polymorphisms, greater than 90% of Caucasians with type 1 diabetes have a HLA DR3 or DR4 allele.[13]

Additional loci associated with, and thought to increase the risk for, type 1 diabetes have been identified and include a variable nucleotide terminal repeat (VNTR) of the insulin gene, polymorphisms of the lymphocyte-specific protein tyrosine phosphatase (*PTPN22*) gene, and cytotoxic T lymphocyte-associated protein 4 (CTLA-4).[14–16] The International Type 1 Diabetes Genetics Consortium (http://www.t1dgc.org), established in 2002, continues to expand the knowledge of the genetic factors that predispose to type 1 diabetes, for example knowledge obtained from genome-wide association studies to identify novel loci that increase risk for type 1 diabetes.[17,18] To date more than 40 loci associated with type 1 diabetes have been identified, including interferon-induced helicase (*IFIH1*), interleukin 2 receptor alpha (*ILR2A*), and three recently identified loci: LIM domain only 7 (*LMO7*), protein EFR3 homolog B (*EFR3B*), and an intergenic region on 6q27.[18–21]

The fact that the risk is not 100% for monozygotic twins of individuals with type 1 diabetes suggests an additional component in the pathogenesis. This prompted the hypothesis that environmental factors are required to trigger islet autoimmunity and initiate the process of islet destruction in genetically predisposed individuals. Specific triggers in human type 1 diabetes are relatively unknown but proposed factors include viral infections, such as coxsackievirus; certain dietary components including gliadin, cereal, and method of infant feeding (breastfeeding versus cow's milk); and improved sanitation and decreased childhood infections, the aptly named "hygiene hypothesis." The exact immunological events leading to insulitis (islet inflammation), beta cell loss, and subsequent diabetes are complex and not entirely understood. A full review of the current knowledge of these events is beyond the scope of this paper. In general, however, both the innate and adaptive immune systems are inappropriately activated and recruited to the pancreas by a triggering event, initiating an immune cascade

that ultimately results in loss of self-tolerance and islet destruction.

Murine models have provided much of the background knowledge into the immunology of type 1 diabetes, though data in humans are growing. In studies of NOD mice, APC, such as macrophages and dendritic cells (DC), are the first to infiltrate the pancreas, presumably returning to pancreatic lymph nodes to present beta cell antigens to naive $CD4^+$ T cells, "priming" these $CD4^+$ T cells, and transforming them into a Th1 subtype.[22,23] Once triggered, autoreactive T cells converge on the pancreas and insulitis ensues. Damage to the islets produces additional self-antigens, which further amplifies T cell activation. B cells are also involved in the pathogenesis of diabetes in the NOD mouse via antigen presentation and production of proinflammatory cytokines as well as production of islet cell antibodies.[24,25] Furthermore, impaired production and action of $Foxp3^+$ regulatory T cells (T_{reg} cells) results in abnormal Th1 and Th2 cell responses.[26,27] Th1 cells subsequently produce additional cytokines, attracting $CD8^+$ cytotoxic T cells that function to initiate cell death or apoptosis of beta cells, which leads to loss of insulin secretion and clinical manifestations of type 1 diabetes.[28]

Although studying type 1 diabetes immunology in humans has proven more difficult, many of the findings to date are similar to those in animal models. Specifically, autoreactive T cells clearly play a prominent role in human type 1 disease development. Studying cadaveric pancreases of individuals with new-onset type 1 diabetes, Wilcox *et al.* revealed that $CD8^+$ cells, along with macrophages, were the predominant cell types in islet infiltrates.[29] Ineffective function of T_{reg} cells is also thought to contribute to disease development in humans.[30,31] Deciphering the triggering and propagating event(s) in the autoimmune cascade could potentially allow for targeted screening, prophylaxis, and therapy for type 1 diabetes.

The intestinal microbiome

Background

Humans are colonized and live in a symbiotic relationship with a vast number and variety of microorganisms, termed the "microbiome," that influence development and general health. The majority of these organisms are bacteria, and it is estimated that the average human microbiome contains 10^{14} bacteria. While these organisms colonize many epithelial surfaces, including skin and upper airways, the intestinal tract, especially the large intestine, harbors the largest number of bacteria.

Early in the neonatal period the microbiome is established and continues to develop over several months to a year toward an adult microbiome. The route of delivery (vaginal vs. cesarean section) and method of nutrition (breastfeeding vs. formula) strongly influence an infant's core microbiota. Vaginal delivery exposes the neonate to the mother's vaginal and intestinal flora. The intestinal microbiome of infants born by cesarean section, however, is initially dominated by skin flora, namely *Staphylococcus*, with delayed acquisition of *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, and *Escherichia coli*.[32–34] The alterations in flora between cesarean section and vaginal delivery persist well beyond infancy.[34] Of particular relevance to this paper, risk of type 1 diabetes onset in childhood is higher in children delivered by cesarean section.[35] Adult microbiota remain relatively stable over time, as reported by Manichanh *et al.*, who showed over two years that an individuals microbiome will maintain phylotypes with 60% similarity.[36]

External factors influence the composition and function of the intestinal microbiome in humans. In particular, dietary exposures likely affect the functional diversity by altering the proportion of various members of the microbiome within the intestine. A poignant example of this phenomenon was presented in a study of twins that revealed obese individuals have reduced diversity of the intestinal microbiome compared to their lean, twin controls.[37] Specifically, the intestinal microbiome of obese individuals had a reduced abundance of Bacteroidetes and an enhanced abundance of Firmicutes and Actinobacteria associated with a reduced functional diversity compared to lean controls. Recently, Muegge *et al.* sampled fecal DNA from 33 mammalian species and 18 humans to understand the effects of diet on a wide range of species and dietary habits.[38] Differences in the structure and function of the intestinal microbiome were influenced by whether the host was an herbivore or carnivore. Interestingly, in the human subjects, who were asked to maintain meticulous food diaries, differences in the structure and function of the intestinal microbiome could be seen among these individuals based on their dietary intake. Taken together, these findings

suggest that dietary exposures can directly influence the diversity and function of the intestinal microbiome that can further affect immune development.

The intestinal microbiome is vast and diverse and the bacterial 16S ribosomal RNA gene sequence (16S) provides a useful tool for analyzing the scope and diversity of the intestinal microbiome. The near ubiquitous expression of 16S in bacteria and the ease of use compared to DNA–DNA hybridization explain the widespread application of 16S gene sequencing in studies of bacterial communities. Utilizing 16S analysis, 395 phylotypes have been identified in the intestinal microbiome, and approximately 80% of these species were not able to be cultured with current methods.[39] Though many phyla are represented, the human intestinal microbiome comprises mainly four phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria, with Firmicutes and Bacteroidetes being the two most prominent phyla.[40,41] Between individuals, however, significant variability exists in the bacterial composition of the intestinal microbiome.[37]

While 16S studies provide information on the number of known species in the microbiome, no information is provided on the function of these bacteria. Metagenomics has evolved as a powerful method to obtain and analyze the genetic diversity of the microbiome. Utilizing a "shotgun sequence" approach, massively parallel pyrosequencing, and complex computer software, metagenomics allows for evaluation of large samples of microbial genes including genes related directly to function. Initial studies utilizing metagenomic analysis of the human intestinal microbiome revealed multiple genes not normally found in the human genome.[42] Many of these gene products were involved in processes of amino acid, glycan, xenobiotic, and vitamin metabolism and biosynthesis. More recently, Qin *et al.* performed metagenomics on a larger cohort of 124 healthy Europeans, revealing the presence of approximately 1,150 bacterial species within the cohort.[43] Each individual's intestinal microbiome was estimated to comprise 160 bacterial species that contributed 150-fold more genes compared to the human gene set.

Despite the variability of microbiota between individuals, a recent metagenomics study of 22 individuals from four countries suggests the existence of "enterotypes," distinct groups of human microbiota that may respond differently to environmental stimuli.[44] The ongoing Human Microbiome Project was developed to form a more concrete understanding of the composition of the human microbiome and the role the microbiome plays in normal physiology and development of disease.[45]

Though much knowledge is gained with metagenomics studies, the methods whereby intestinal bacterial samples are collected and analyzed must be emphasized and standardized, as variable methods can yield inconsistent results. Colonic bacteria samples collected from the same individual via a stool sample or intestinal biopsy may reveal different bacteria.[39] Methods used to extract samples may also influence the bacterial composition data.[46] Momozawa *et al.* recently confirmed the differences seen in bacterial yield between stool and biopsy samples, and that methods of stool sample recovery, either from colonoscopy or fresh stool sample, can identify different bacteria.[47] The authors contend that only biopsy specimens should be used for high-throughput analysis of human colonic bacteria.

Intestinal microbiome and autoimmunity

The intestinal microbiome is a complex system that acts in a symbiotic relationship with the host to influence development, nutrition, immunity, and disease. The intestinal mucosa is a common entry site for pathogens and harbors a significant proportion of the cells of the immune system. An intact mucosa provides the first line of defense against pathogens and other pathogenic antigens. Research in animal models and humans continues to define the vital role the intestinal flora plays in protecting the mucosa from invading pathogens and influencing the development and maintenance of both the systemic and innate immune systems.

Epidemiological data have revealed a disproportionate prevalence of autoimmune diseases in developed countries. This constitutes the original basis for the "hygiene hypothesis," the suggestion that decreased exposure to microorganisms, both pathogenic and symbiotic, in childhood alters natural development of the immune system predisposing to the loss of self-tolerance.[48] This hypothesis has fostered the concept that altered intestinal microbiota may be one of the predisposing factors for the development of autoimmunity. In addition to the knowledge that intestinal microbiota contribute significantly to the development and maintenance of the immune system, recent animal model and

human studies of several different autoimmune diseases lend additional credence to the claim that altered intestinal flora may be at the forefront of the pathogenesis of these diseases.

Crohn's disease and ulcerative colitis, known broadly as inflammatory bowel disease (IBD), are autoimmune diseases of the intestinal tract that lead to mucosal inflammation and development of intestinal lesions. IBD is also associated with several extraintestinal manifestations. The exact etiology of IBD is unclear but likely involves environmental, genetic, and immune factors. The role of the microbiome in IBD development is becoming more established. Animal studies have revealed that the severity of experimentally-induced intestinal inflammation can be modulated via introduction of anaerobic bacteria.[49] Interestingly, germ-free animals appear to be protected from experimentally induced colitis but show rapid development of disease upon colonization with enteric bacteria.[50–52] Metagenomic evaluation of fecal samples from patients with Crohn's disease has revealed a reduced diversity of the Firmicutes phyla compared to healthy controls.[53] Similarly, Frank *et al.* characterized subsets of patients with Crohn's disease and ulcerative colitis who had reduced bacteria in the Firmicutes and Bacteroidetes phyla.[54] Willing *et al.* provided further evidence of an altered intestinal microbiome in Crohn's disease in their study of monozygotic twins.[55] Small intestine biopsies of twins concordant or discordant for ileal Crohn's disease revealed a preponderance of *E. coli* and a reduced abundance of *Faecalibacterium prausnitzii*, compared to individuals with colonic Crohn's or healthy controls.

Alterations in intestinal flora have been hypothesized to contribute to the pathogenesis of several other autoimmune diseases including celiac sprue, allergy, multiple sclerosis, rheumatoid arthritis, and ankylosing spondylitis.[56–60] Identifying specific intestinal microorganisms that alter risk of a disease will not only assist in defining pathogenesis but also provide a method of screening and the ability to tailor therapy specifically.

The intestinal microbiome: effects on immunity and risk of autoimmune diabetes

Much of the knowledge regarding the role that the intestinal microbiome may play in the development of autoimmune diabetes comes from animal studies using diabetes-prone and germ-free animals. These studies, combined with epidemiological data from humans, have begun to establish the many facets of the intestinal microbiome that directly affect the risk for, and development of, autoimmune diabetes. Additionally, early therapeutic studies have also been established directly targeting the intestinal microbiome. As illustrated in Figure 1, several environmental factors thought to be triggers of autoimmune diabetes may, at least in part, increase risk for diabetes due to their effects on the composition of the intestinal microbiome.

Animal studies

Animal models have provided a wealth of information regarding the influence of the intestinal flora on autoimmunity and autoimmune diabetes development.

Alterations in flora. Using mice raised in germ-free environments and subsequently exposed to microorganisms of choice (gnotobiology), much has been ascertained about the structural and functional effects of the intestinal flora on the immune system. Peyer's patches, splenic germinal centers, and mesenteric lymph nodes of germ-free mice are significantly smaller and fewer in number compared to mice raised in a normal environment.[61] Deficiencies in the development of mucosal-associated lymphoid tissue, specifically plasma cells and $CD4^+$ T cells, are other consequences of a germ-free environment.[62,63] In germ-free animals, reintroduction of normal gut flora can normalize size and cellularity of lymphoid structures and increase antibody production.[64] In fact, colonization of germ-free mice with a single organism, segmented filamentous bacterium (SFB), is sufficient to stimulate production of IL-17–producing $CD4^+$ T cells.[65] The intestinal microbiome affects several other components of immune development and function, as will be outlined throughout this section.

The sentinel studies suggesting a role for microorganism exposure in the pathogenesis of autoimmune diabetes employed diabetes-prone animals, NOD mice, and BioBreeding diabetes-prone (BB-DP) rats in particular, raised germ-free and/or exposed to a variety of infectious organisms or antigens.

Early studies revealed that in NOD mice, chronic viral infections were associated with a lower incidence of diabetes.[66,67] Active infection with mycobacteria and stimulus with bacterial antigens also

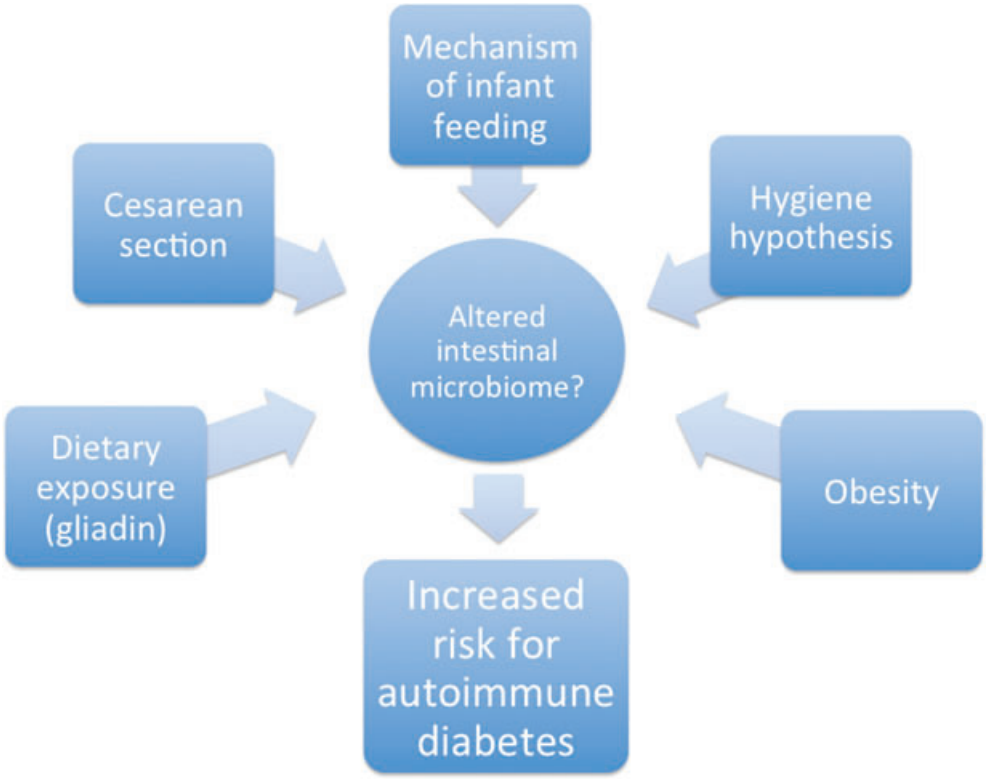

Figure 1. Environmental factors that may directly or indirectly alter the intestinal microbiome and therefore affect risk of autoimmune diabetes.

decreased the rate of development of autoimmune diabetes in NOD mice.[68–71] Extrapolation of these findings has led to the concept that a germ-free environment specifically increases the risk of diabetes development in NOD mice. However, we have shown that diabetes incidence in female NOD mice is not necessarily affected by germ-free conditions; rather, monoculture of these animals with *Bacillus cereus* delayed onset of diabetes, suggesting specific bacteria or bacterial products affect the risk of disease more so than a specific germ-free environment.[72] The protective effect of bacterial extracts may require functional natural killer cells (NKC) and involve increased production of TGF-β.[73] The ability to stimulate T_{reg} cell production, through induction of FoxP3 expression, may explain the protective effect of TGF-β.[74] Controversy exists over the protective role of NKC in autoimmune diabetes, however, as these cells have also been implicated in the pathogenesis.[75]

Identifying specific organisms associated with the development of autoimmune diabetes would allow for targeted screening and potential therapies. Initial attempts to identify specific intestinal flora alterations that increased risk for type 1 diabetes used diabetes-prone animal models. Performing fluorescent *in situ* hybridization to target 16S rRNA of *Bacteroides*, *Clostridium*, and *Lactobacillus* species in fecal samples of BB-DP rats, Brugman *et al.* revealed that *Bacteroides* sp. were more prominent in those rats that developed diabetes compared to rats that remained diabetes free.[76] More in-depth experiments revealed significantly higher proportion of *Lactobacillus* and *Bifidobacterium* genera in biobreeding diabetes-resistant (BB-DR) rats compared to BB-DP rats, while BB-DP rats were found to have a higher abundance of *Bacteroides* genera.[77]

Innate immunity and intestinal integrity. The innate immune system relies on the microbiome for appropriate development of several cell types. Of particular interest is the intestinal epithelial cell (IEC). IECs contain tight junctions that normally function to regulate passage of nutrients and inhibit

translocation of pathogenic organisms. IECs also express direct interaction with immune cells and produce and respond to a variety of cytokine stimuli. Many changes in IEC development and function are observed when alterations are made to the intestinal flora of animal models. For example, IECs of germ-free mice turn over at a slower rate than mice with normal intestinal microflora.[78] Additionally, mice depleted of their normal flora by antibiotics also show diminished IEC replication, significant alteration in IEC gene expression, and impaired production of antimicrobial factors including RegIIIγ, a Gram-positive specific antimicrobial peptide.[79] Reintroduction of colonic bacteria induces proliferation of IECs in germ-free mice via toll-like receptor (TLR) recognition of the microbes.[80]

IECs play a prominent role in the development and regulation of the immune system. An intact intestinal epithelium serves to regulate passage of antigens to DC and increased gut permeability due to a compromised epithelium results in exposure to antigens that is a potential trigger for an autoimmune response in predisposed individuals. IECs also function to uptake, process, and present antigen and can promote activation of $CD8^+$ T cells, as reviewed.[81] Additionally, IEC modulate function of DC via production of factors such as IL-10 that promote a tolerogenic DC response.[82] IEC may also play a role in T_{reg} cell expansion.[83] Taken together, this evidence suggests that IEC are critical to the proper development of the innate immune system and dysregulation of IEC by an altered intestinal flora may result in the development of autoimmunity.

Evidence in the BB-DP rat model has suggested that *Lactobacillus johnsonii* N6.2 is a specific organism that may delay the onset of autoimmune diabetes via modulation of intestinal integrity.[84] In their study, Valladares *et al.* also noted a significant increase in the tight junction protein claudin-1 and decreased oxidative stress in the ileal mucosa of the *L. johnsonii* treated animals. These findings suggest that autoimmune diabetes may be the result of an inflammatory response initiated by a leaky gut epithelium, which is the result of an altered intestinal flora.

Further evidence from animal studies suggests that the integrity of the gut epithelium plays a prominent role in autoimmune diabetes development. BB-DP rats express less of the tight junction protein claudin and display increased intestinal permeability compared to control Wistar rats.[85] Translating further, increased gut permeability was associated with a higher rate of diabetes development in BB-DP rats on a regular diet.[86] Watts *et al.* additionally demonstrated that upregulation of zonulin—a protein that serves to regulate tight junctions—and, subsequently, increased intestinal permeability significantly increased the rate of diabetes development in BB-DP rats.[87]

More recently, Wen *et al.* sought to establish how the interaction between microorganisms and the intestinal epithelium could trigger autoimmunity and diabetes development.[88] The specific target of this study was myeloid differentiation factor 88 (MyD88), an adaptor molecule used by multiple TLR to regulate the innate immune response. Wild-type NOD mice developed diabetes quickly. NOD mice with a MyD88 knockout (KO), however, were protected from diabetes development while germ-free, MyD88-KO NOD mice developed diabetes in a rapid fashion. Furthermore, recolonization of the MyD88-KO mice decreased diabetes development, suggesting protection from diabetes is microorganism dependent. Knockdown of TLR2, TLR3, and TLR4 did not attenuate diabetes development, however, suggesting that while some microorganisms protect the host from developing diabetes, this protection is independent of the TLR system.

Adaptive immune system. The development and function of the adaptive immune system is also directly affected by the intestinal microbiota, a process that may influence risk of disease. T_{reg} cells secrete anti-inflammatory cytokines IL-10 and TGF-β and function to temper the immune response through down-regulation of both the innate and adaptive immune systems.[89] Intestinal T_{reg} cells in particular play a prominent role in tolerance of oral antigens and microbes.[90] Evidence from animal models suggests the presence of the intestinal microbiota is vital for the production of adequate numbers of functional T_{reg} cells from the intestine. In germ-free mice, T_{reg} cells are less prominent and less effective in suppressing T cell activity compared to T_{reg} cells in conventional mice.[91] In an experimental model of colitis, IL-10 production from T_{reg} cells of germ-free mice is also suppressed, as is the ability of these cells to modulate disease.[92] Conversion of $CD4^+$ T cells to T_{reg} cells was stimulated by *B. fragilis* monocolonization of germ-free mice, a process dependent on

the *B. fragilis*-produced polysachharide A (PSA).[93] IL-10 production and T cell suppressive activity of T_{reg} cells were also enhanced in a PSA-dependent fashion.

T helper 17 cells (Th17) are a proinflammatory subset of T cells that secrete interleukin 17 (IL-17), have antimicrobial activities, and have been implicated in many autoimmune diseases including multiple sclerosis, inflammatory bowel disease, and psoriasis.[94] The intestine harbors the largest number of Th17 cells, and the intestinal microbiota is likely required for the production and expansion of these cells.[95–97]

In a recent report, Lau *et al.* sought to understand the mechanisms by which *L. johnsonii* exposure can delay diabetes in BB-DP rats.[98] Interestingly, the study demonstrated enhanced Th17 differentiation in the mesenteric lymph nodes of animals fed *L. johnsonii*. The authors suggested that although Th17 cells are associated with insulitis the Th17 bias seen with *L. johnsonii* feeding limits the necessary conversion to the diabetogenic Th1 cell type, thereby inhibiting or delaying onset of diabetes. Others have suggested that a Th17 bias may reduce the risk of autoimmune diabetes via mucosal protection produced by IL-17 upregulation.[99] Other undefined mechanisms may also explain the effect seen with *L. johnsonii* exposure.

Other important regulatory cells of the immune system are directly influenced by the intestinal microbiome, including DC, which do not develop in appropriate numbers in germ-free mice, and B cells, which produce IgA at a reduced amount in germ-free mice, compared to wild-type mice.[100,101]

Dietary factors. The intestinal microbiota's influence on nutrition and the potential role of these factors in autoimmune diabetes pathogenesis has also been explored in animal models. The initial evidence suggesting a role for nutrition in autoimmune diabetes came from Hoorfar *et al.* in 1993.[102] Using NOD mice exposed to wheat-flour diet or hydrolyzed casein, this study revealed a significantly lower incidence of diabetes in mice receiving the hydrolyzed diet. Expanding further, Brugman *et al.* completely prevented diabetes by providing antibiotics and a hydrolyzed casein diet to BB-DP rats.[76]

Additional studies have expanded on the dietary hypothesis to more precisely pinpoint the components and associated mechanisms of diet that may increase risk for autoimmune diabetes. Gliadin, a glycoprotein implicated in the intestinal damage of celiac sprue, is the most extensively studied dietary component in the pathogenesis of type 1 diabetes. NOD mice that lack exposure to dietary gluten develop diabetes at a significantly lower rate than mice fed a standard diet.[103–105] BB-DP rats fed a cereal-based diet develop diabetes associated with a pancreatic Th1 cytokine pattern, compared to BB-DP rats on a protein-based diet who had less insulitis, a Th2 cytokine pattern, and overall lower incidence of diabetes.[106] Investigation into the mechanisms leading to development of insulitis and diabetes in animals on a gliadin diet suggests that these proteins increase small intestine inflammation and intestinal permeability.[86,107] Furthermore, BB-DP rats have a significantly higher proportion of Th1 cells in the mesenteric lymph nodes upon exposure to wheat proteins.[108] Pancreatic lymph node dendritic cells sample gut antigens and, upon recognizing protein products, stimulate production of activated T cells, a process that leads to increased beta cell apoptosis.[109] Finally, gliadin exposure also suppresses T_{reg} cell production in NOD mice, which is another potential mechanism by which dietary exposure may enhance genetic diabetes risk.[110]

The known association of alterations in diet and intestinal flora with diabetes development suggests that these two entities work in concert to affect disease risk. With this in mind, the interactions between intestinal bacteria and gliadin peptides are being closely examined. Hansen *et al.* examined the effect of a gluten-free versus standard diet on bacterial composition in NOD mice.[111] Diabetes developed in 47% of the standard-fed mice compared to only 5% in the mice fed a gluten-free diet. Examination of intestinal bacteria revealed a significantly lower prevalence of aerobic, microaerophilic, and anaerobic bacteria in the mice fed a gluten-free diet, compared to mice on a standard diet. Much of the difference in bacterial composition was directly attributable to Gram-positive bacteria.

In vitro studies have provided additional information regarding the interactions between gliadin and intestinal flora. As shown by Laparra *et al.*, intestinal epithelial cells (Caco-2) in culture exposed to gliadin-derived peptides produce inflammatory cytokines, a process that was downregulated when these cell preparations were inoculated with *Bifidiobacteria*, an intestinal bacteria that has been

associated with lower incidence of diabetes in animal models.[112] The effect was most pronounced with *Bifidobacterium longum*. Gliadin peptide sequences were modified upon exposure to *B. longum*, suggesting a mechanism by which "protective" intestinal bacteria can indirectly maintain intestinal integrity. The same research group recently examined the proteome of Caco-2 cells after exposure to gliadin peptides in the presence or absence of *B. longum*.[113] Protein expression was altered in Caco-2 cells treated with gliadin peptides in the absence of *B. longum*; specifically, expression of proteins involved in cytoskeletion formation and apoptosis was altered. These effects were ameliorated when *B. longum* was added to the cell culture.

Whereas *B. longum* may protect the intestinal epithelium from gliadin-induced structural changes, other species of bacteria have been shown to act with gliadin to synergistically alter the integrity of the intestinal epithelium. Germ-free rats exposed to gliadin were found to have fewer goblet cells in the small intestine, a sign of early enteropathy, and this finding was more pronounced in animals inoculated with *Escherichia coli* CBL2 or *Shigella* CBL2.[114] *Shigella* CBL2 also augmented interferon-γ–induced impairment of tight junctions, allowing enhanced translocation of gliadin into the lamina propria.

The results from the above studies indicate that gliadin exposure increases the risk for diabetes through mechanisms involving both the integrity on the intestinal epithelium and the composition of the intestinal flora.

Probiotics. Expanding further on the concept of a protective intestinal flora, modification of the intestinal flora by probiotics has been investigated as a method to modulate the risk for diabetes. *Lactobacillus casei*-treated NOD mice were protected from diabetes onset and were found to have reduced numbers of splenic $CD8^+$ T cells and systemic inflammatory markers.[115] IL-2 levels were also higher in probiotic-treated mice, and the enhanced expression of this cytokine may serve to stabilize $FoxP3^+$ T_{reg} cells, as recently described by Chen *et al.*[116] NOD mice administered the probiotic compound VSL#3 showed a reduced severity of insulitis, reduced beta cell destruction, and lower rates of diabetes development compared to control.[117] In this study, IL-10 expression was significantly increased in the Peyer's patches, spleen, and pancreas, suggesting an immunomodulatory effect of the probiotic. Another potential mechanism behind the protective effect of probiotics may include these organisms' ability to inhibit adherence of enteropathogenic bacteria by binding to intestinal epithelial cells and upregulating mucin production.[118,119]

Human studies

Using discoveries in animal models, studies are beginning to emerge establishing the role of intestinal flora and integrity in the development of autoimmune diabetes in humans.

Alterations in flora. Attempts have been made to define an understanding of the underlying intestinal flora alterations underlying autoimmune diabetes development in humans. Using 16S RNA amplification techniques, Giongo *et al.* set out to define specific taxa that differed between children with type 1 diabetes and healthy controls.[120] Over time, the intestinal microbiota of children who developed type 1 diabetes consisted of a higher proportion of the Bacteroidetes phyla and a lower proportion of the Firmicutes phyla. Opposite findings were observed in control patients, in whom Bacteroidetes phyla sequences decreased over time while Firmicutes sequences increased. Additionally, within the Bacteroidetes phyla, the *Bacteroides ovatus* species represented 24% of the total increase in cases. Though this study was small, comprising eight case subjects and four controls, the findings represented some of the first evidence of specific changes in the composition of intestinal bacteria in humans with type 1 diabetes. Larger cohort studies such as The Environmental Determinants of Diabetes in the Young (TEDDY, currently in progress), are needed to further define alterations in the composition of the intestinal microbiome in humans and the underlying mechanisms that lead to autoimmunity and diabetes development.[121]

Intestinal integrity and immunity. Similar to animal models of autoimmune diabetes, several studies of intestinal integrity in human subjects with type 1 diabetes have revealed evidence of increased intestinal permeability.[122–124] Furthermore, the intestinal permeability seen in individuals with type 1 diabetes is detectable prior to clinical onset of disease, suggesting that the intestine is directly involved in the disease development.[125] A recent study by Brown

et al. used metagenomics to determine the potential function of bacteria that differ between individuals with type 1 diabetes and healthy controls.[126] Findings from this study may begin to explain the contribution of the intestinal microbiome to maintenance of intestinal integrity. Bacteria from individuals with type 1 diabetes tended to have higher expression of genes related to motility and adhesion compared to controls. Additionally, evaluation of 16S demonstrated a higher proportion of butyrate-producing and mucin-degrading bacteria in controls compared to cases. Both butyrate and mucin are thought to be directly involved in maintaining intestinal epithelial integrity. The authors suggest a hypothesis whereby healthy individuals harbor a higher proportion of butyrate-producing bacteria that help to maintain intestinal integrity and prevent autoimmunity. These preliminary findings may help to advance the knowledge of the role of intestinal integrity in autoimmunity and type 1 diabetes development.

Inflammatory changes in the intestine of patients with type 1 diabetes lend further evidence for the involvement of the intestinal mucosa and altered intestinal immunity in the pathogenesis of type 1 diabetes. Though sometimes structurally normal, small intestine biopsies of patients with type 1 diabetes reveal increased expression of HLA-DR, HLA-DP, and intercellular adhesion molecule-1 (ICAM-1).[127] Frequently, microstructural intestinal changes are seen in patients with type 1 diabetes, including changes to the microvilli and tight junctions.[128] Higher densitites of IL-1α^+ and IL-4$^+$ cells in these biopsies also point toward a heightened intestinal inflammatory state in type 1 diabetes. Jejunal biopsies of patients with type 1 diabetes show evidence of mucosal inflammation and, upon *in vitro* exposure to gliadin, an exaggerated inflammatory response compared to control.[129] Finally, T_{reg} cell production in the intestinal mucosa is suppressed in patients with type 1 diabetes, as demonstrated by reduced numbers of FoxP3$^+$ cells in small intestine biopsies of children with type 1 diabetes.[130]

Dietary factors. As in animal models, human intestinal integrity appears to be compromised upon exposure to gliadin peptides. Human intestinal samples exposed to gliadin *ex vivo* display amplified release of zonulin and increased permeability.[129] Intestinal samples from patients with celiac disease had more robust and sustained permeability compared to nonceliac disease controls, consistent with the theory of genetic predisposition enhancing risk in those with celiac disease. The role of gliadin in the pathogenesis of type 1 diabetes likely goes beyond affecting intestinal integrity. Small bowel biopsies of patients with type 1 diabetes exposed to gliadin reveal an exaggerated inflammatory response.[129] Production of cytokines TNF-α and IL-8—both involved in the pathogenesis of type 1 diabetes—by monocytes of susceptible humans is also stimulated by gliadin.[131] Finally, islet cell autoimmunity appears to be exaggerated in individuals who are exposed to gliadin-containing foods prior to six months of age.[132] Given the association of type 1 diabetes and celiac disease, and the role of gliadin in both, it is likely that these disease processes share specific risk factors and etiologies. Similar genetic susceptibility between the two diseases, as revealed by Smyth *et al.*, further enhances the concept of shared pathogenic mechanisms.[133]

Probiotics. Based on initial promising results in animal models, the use of probiotics to delay or prevent type 1 diabetes in humans has become an area of interest. Studies in healthy humans have demonstrated that exposure to *Lactobacillus plantarum* enhanced the expression of tight junction proteins,

Table 1. Organisms and other factors that may alter risk for autoimmune diabetes in diabetes-prone animal models and humans

May delay or prevent autoimmune diabetes	May promote autoimmune diabetes
Chronic viral infections[a]	*Bacteroides* sp.[a,b]
Mycobacterial infection[a]	*Bacteroides* genera[a,b]
Bacterial antigens[a]	Gliadin[a,b]
Monoculture with *B. cereus*[a]	Impaired intestinal epithelial integrity[a,b]
Lactobacillus genera[a,b]	Cow's milk[b]
Lactobacillus johnsonii N6.2[a]	Delivery by cesarean section[b]
Lactobacillus casei[a]	Hygiene hypothesis[b]
Probiotic VSL #3[a]	Acute viral infections[b] (coxsackievirus)
Bifidobacterium genera[a]	

[a]Animal models, [b]humans.

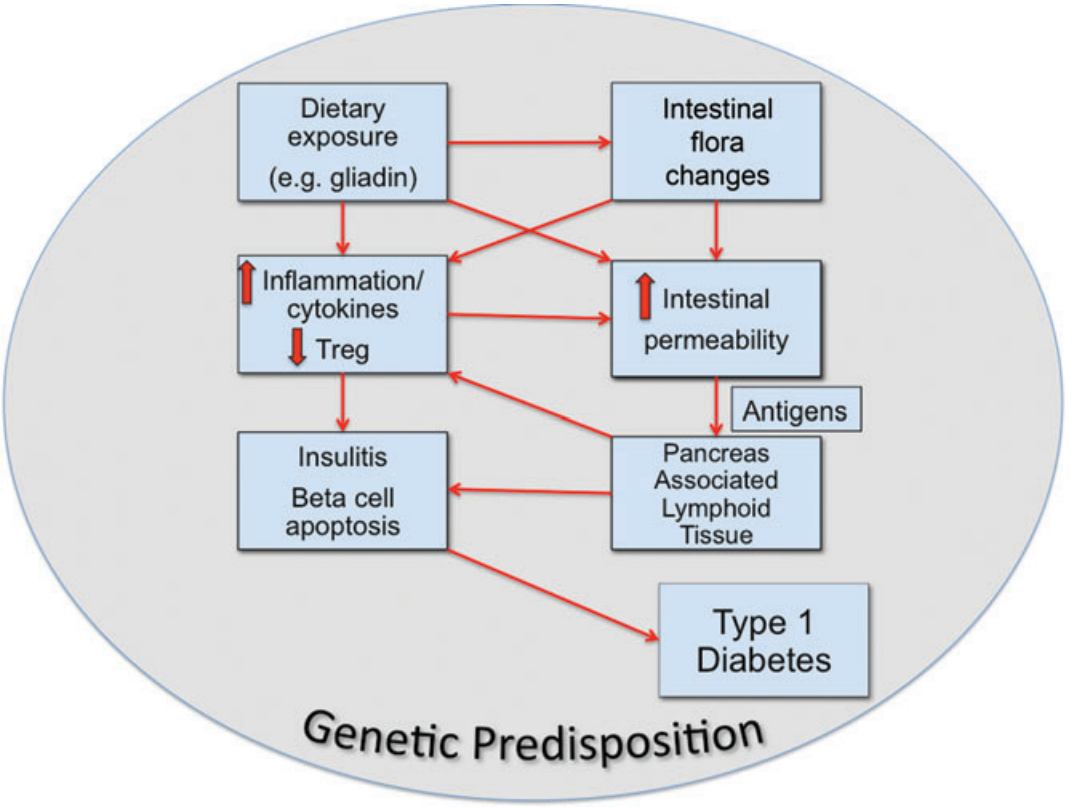

Figure 2. General schema outlining proposed relationships between intestinal flora/integrity and development of type 1 diabetes in genetically predisposed individuals.

suggesting the ability of these organisms to improve endothelial integrity.[134] The PRODIA study, currently ongoing in Finland, is investigating children with genetic susceptibility to type 1 diabetes and the ability of probiotics to decrease diabetes autoantibodies in this group.[135]

Conclusion

Type 1 diabetes is a complex series of events culminating in autoimmune destruction of pancreatic beta cells, hyperglycemia, and risk for subsequent vascular complications. In those with genetic predisposition, environmental factors affect disease risk and pathogenesis. Table 1 briefly summarizes some of the specific organisms and factors that have been identified to potentially alter risk of autoimmune diabetes development in diabetes-prone animal models and humans. Evidence to date strongly suggests that intestinal microbiota, through their impact on immune development and intestinal structure and function, are vital to the pathogenesis of type 1 diabetes, though the exact mechanisms whereby intestinal bacteria are altered and how those alterations influence type 1 diabetes development are still unclear. Figure 2 presents a general view of the knowledge to date regarding potential mechanisms in the development of type 1 diabetes in genetically predisposed individuals and how the intestinal microbiome may play a direct role in this process. Continued exploration into the specific role of the intestinal microbiome in the development of type 1 diabetes will allow for targeted screening and development of novel therapies to treat and ideally prevent the disease.

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Conflicts of interest

The authors declare no conflicts of interest.

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