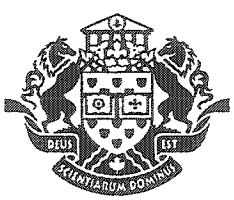

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Chronic Oral Administration of IL-10-Secreting *Lactococcus lactis* to Diabetes-Prone Rats

by

Timothy David Wallace

Thesis

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Chronic oral administration of IL-10-secreting *Lactococcus lactis* to diabetes-prone rats

Type 1 Diabetes Mellitus (T1D) is an autoimmune disease characterized by the destruction of insulin-producing beta cells in the pancreas. This autoimmune response arises because of poorly understood interactions between genetic and environmental factors. A strong link has been observed between diet and diabetes outcome. There is also evidence that T1D patients exhibit increased gut permeability and inflammation, characterized by a Th1 cytokine bias. This suggests that T1D may be gut-related. Oral delivery of IL-10-secreting bacteria has been shown to counteract Th1-mediated colitis in mice. To test whether a similar treatment might affect diabetes development, diabetes-prone BB (BBdp) rats were gavaged daily with 2×10^9 CFU of mIL-10-secreting *Lactococcus lactis* pTRmIL-10. Treatment was initiated at ~26 days and terminated at 160 days or upon diagnosis of diabetes, after which animals were sacrificed. Erythromycin-selective plate counts showed that pTRmIL-10 and pTR1NX (control plasmid) were present in all segments of the gastrointestinal tract with higher levels observed in the distal small intestine and colon. Diabetic animals exhibited increased *L. lactis* levels compared to control and end-of-study animals, suggesting that *L. lactis* growth was altered in overt diabetic animals. Analysis from a previous study showed that *Lactobacillus*, *Staphylococcus* and *Enterococcus* species, as well as total Coliforms were also increased in the colon of diabetic animals. This suggests that overgrowth of the gut microflora may result at onset of overt diabetes. A trend towards increased disease incidence was observed in animals treated with L. lactis pTRmIL-10 compared to those treated with pTR1NX and control *L. lactis* p663 (86.5%, 68% and 68% incidence, respectively) although this difference was not statistically significant. Serum antibodies from animals gavaged with pTRmIL-10 were reactive against proteins produced by pTRmIL-10 but not against commercial mIL-10. This suggests that a humoral immune response against constitutive proteins of pTRmIL-10 may have contributed to an increase in diabetes incidence in animals treated with pTRmIL-10 when compared to the historical rate of the disease. Semi-quantitative RT-PCR analysis showed that oral delivery of pTRmIL-10 did not alter the cytokine profile towards a Th2 response in the gut mucosa. Further analysis suggested that murine mIL-10 did not transit the mucosal barrier as no mIL-10 was detectable in the serum of animals treated with pTRmIL-10. It is unclear if this reflects minimal mIL-10 production *in vivo* or lack of species specificity. Rat IL-10 was undetectable in the serum of control and pTRmIL-10-treated animals. In conclusion, chronic oral exposure of BBdp rats to bacteria engineered to secrete biologically active mIL-10 resulted in an increase in diabetes frequency when compared to animals treated with buffer alone but not compared to animals fed control bacteria. This increase is attributed to reactivity to proteins constitutively secreted by the mIL-10 producing vector. No change was observed in the Th1/Th2 cytokine profile in the proximal duodenum of these animals.

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Abbreviations

-/-	double-knockout
AV	Adeno-virus
AAV	Adeno-associated Virus
ATCC	American Type Culture Collection
BBc	Biobreeding control rat, Ottawa colony
BBdp	Diabetes-prone Biobreeding rat, Ottawa colony
BBDP	Diabetes-prone Biobreeding rat, Worcester colony
BBDR	Diabetes-resistant Biobreeding rat, Worcester colony
BBd	Overt diabetic Biobreeding rat, Ottawa colony
BB/OK	Biobreeding rat, Ottawa-Karlsburg colony
BLG	Beta-lactoglobulin
BM9	Sodium-bicarbonate buffered M9 minimal salts medium
CD	Crohn's Disease
CFU	Colony Forming Units
CMV	Cytomegalovirus
DSS	Dextran Sulfate Sodium
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
GALT	Gut associated lymphoid tissue
GI	Gastrointestinal
GRAS	Generally Regarded as Safe
hIL-10	Human Interleukin-10
HLA	Human Leukocyte Antigen
IAN-5	Immune Associated Nucleotide-5
IBD	Inflammatory Bowel Disease
IEC	Intestinal epithelial cell
IEL	Intestinal epithelial lymphocyte
IFN-γ	Interferon-gamma
IL-	Interleukin-
iNOS	Inducible Nitric Oxide Synthase
KRV	Kilham Rat Virus
LAB	Lactic Acid Bacteria
M17	*Lactococcus lactis* growth medium
MAdCAM-1	Mucosal vascular addressin
MHC	Major Histocompatibility Complex
MLN	Mesenteric lymph nodes
NOD	Non-obese diabetic
P663	*Lactococcus lactis* p663
PBMC	Peripheral blood mononuclear cells
pDNA	Plasmid DNA
PP	Peyer's patches
PTR1NX	*Lactococcus lactis* pTR1NX
PTRmIL-10	*Lactococcus lactis* pTRmIL-10

SI	Small intestine
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
Th	T-helper
T_{reg}	T regulatory lymphocytes
vIL-10	Viral Interleukin-10

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Introduction

Type 1 Diabetes Mellitus (T1D) is an autoimmune disease that results when insulin-producing beta cells in the pancreatic islets of Langerhans are destroyed by infiltrating immune cells. One of the most common diseases of childhood, it exhibits a mean age of onset of 13 years and involves complex interactions between genes and environmental factors (Melton et al, 1983). Disease incidence exhibits wide geographic variability, ranging from 0.1/100,000 per year in the Zunyi region of China to 49/100,000 per year in certain regions of Finland (Karvonen, et al, 2000).

The destruction of pancreatic beta cells leads to insulin deficiency and impaired cellular uptake of glucose. The resulting elevation in blood glucose levels leads to rapid weight loss, polydipsia, polyuria and fatigue. Clinical symptoms of T1D include hyperglycemia, glucosuria, hypoinsulinemia, ketonuria and ketoacidosis, the last of which is fatal unless oral insulin is administered to regulate blood glucose levels (Hanenberg et al, 1989; Tafuri et al, 1993). Long-term complications of the disease include retinopathy, nephropathy, neuropathy, amputation, increased risk of cardiovascular disease and sterilization.

T1D Pathogenesis

Although exact mechanisms remain unclear, islet destruction appears to result from infiltration into the islets by mononuclear cells, a process called insulitis. Insulitis begins with infiltration of macrophages, mononuclear cells, and possibly dendritic cells. This is followed closely by $CD4^+$ and $CD8^+$ T cells, NK cells, and to a lesser extent, B cell transit into the islets (Hanenberg et al, 1989; Tafuri et al, 1993).

The process that results in T1D is characterized by a predominance of the Th1 $CD4^+$ T helper (Th) cell population in the pancreas. Although it is not clear what triggers the initial polarization of naïve Th0 cells to Th1 or Th2 subsets, the pre-diabetic and diabetic pancreas exhibits a pro-inflammatory Th1 population. This Th1 bias is associated with increased expression of pro-inflammatory cytokines including IFN-γ, TNF-α and IL-12p40 and a corresponding down-regulation of anti-inflammatory Th2 cytokines such as IL-4 and IL-10 (Zipris, 1996; Kolb et al, 1996; Suarez-Pinzon, et al, 2001; Scott et al, 1997).

β-cell destruction is a chronic process occurring over a period of weeks to years. Clinical detection of early-stage insulitis and β-cell destruction is particularly difficult given that symptoms do not arise until shortly before onset of overt diabetes. In diabetes-prone BB (BBdp) rats, an animal model of spontaneous T1D, response to glucose challenge has been shown to be normal within 10 days of disease onset but this response is gradually impaired as β-cell destruction nears completion. This suggests that residual β-cells are capable of increased insulin production sufficient to maintain normal blood glucose levels. Thus, clinical onset occurs when the β-cell population is reduced to a threshold (~10%) incapable of producing sufficient insulin to maintain normal blood glucose (Teruya et al, 1993).

T1D and Genetic Susceptibility

T1D is a polygenic disorder involving at least 20 susceptibility genes. Human monozygotic twins exhibit a concordance rate of 30-40% (Redondo et al, 1999; Redondo et al, 2002). Furthermore, siblings exhibit a concordance of 6% compared to a 0.4% incidence rate observed in the general population (Karvonen et al, 2000).

Genetic susceptibility in humans has been associated with 20 chromosomal regions. The IDDM1 locus is most strongly implicated in disease risk. This locus, located on chromosome 6p21.3 encodes for the Human Leucocyte Antigen (HLA) Class II MHC complex (Park, et al, 1998; Zeigler, et al, 1991). Estimates suggest that MHC Class II region genes provide 40-50% of familial clustering (Risch, 1987; Noble, et al, 1996). IDDM2, a non-coding region of the insulin gene mapped to chromosome 11p15.5 accounts for approximately 10% of disease susceptibility. Polymorphisms within this region are associated with increased diabetes risk through influence on thymic insulin expression (Redondo, et al, 2002). IDDM3-IDDM18 have also been linked to the disease. However, only 10 of these genes have been shown to exhibit statistically significant linkages to the disease (Pociot, et al, 2002). Thus, it is likely that any increased susceptibility attributed to these genes is exerted on an individual or geographic basis but not by an entire population (Davies, et al, 1994).

The BBdp rat model of T1D exhibits many similarities to the human form of the disease. First discovered in an outbred colony of Wistar rats housed near Ottawa in 1975, selective inbreeding has led to the development of a number of genetically distinct colonies. Located throughout the world, disease incidence varies from ~60% in the Ottawa colony to >90% in the BB/Wor strain housed in Worcester, Massachusetts (Mordes et al, 1996).

Similar to the human form of the disease, T1D in the BBdp rat is a polygenic disorder with multiple genetic loci contributing to disease susceptibility. Genetic predisposition is strongly linked to the Major Histocompatibility Complex (MHC) class

II allele (*Iddm2*) which is homologous to the human IDDM1 HLA Class II allele (Mordes et al, 1996; Markholst et al, 1991).

A second susceptibility locus, the *Iddm1* autosomal recessive Lyp gene, maps to chromosome 4 and is present in all BBdp rats. This gene produces a severe lymphopenia resulting in the loss of >85% of $CD8^+$ T cells and a 50-80% reduction in the number of $CD4^+$ T cells. Recently, it has been demonstrated that this lymphopenia is associated with a frameshift deletion in the Immune-Associated Nucleotide (IAN)-5 gene, resulting in truncation of the IAN-5 protein (MacMurray et al, 2002). As IAN-5 expression is upregulated during thymic T-cell development and is expressed on mature T cells, it has been suggested that impaired T-cell development caused by truncation of the IAN-5 protein may be responsible for lymphopenia (MacMurray et al, 2002).

Three more susceptibility genes have also been implicated in disease prevalence. *Iddm3*, first discovered in Fischer rats, has been shown to provide resistance towards the disease (Mordes et al, 2000). Further studies with the BB/OK strain as well as a backcross between BBDP and control Wistar Furth animals have shown that *Iddm5*, linked to chromosome 13, appears to provide protection against the disease (Kloting et al, 1998; Martin et al, 1999). A third locus, *Iddm4* has been mapped to chromosome 13 and has been linked to the *Iddm1* Lyp gene. The location of *Iddm4* is of particular interest given that it is found in a region containing other autoimmune-associated genes with links to arthritis and uveitis (Martin, et al, 1999).

Environmental Triggers of T1D

Although it is well documented that genetic predisposition and immune-dysregulation are contributing factors to disease susceptibility, the rapid increase in T1D

incidence observed over the past half-century suggests that environmental factors play a pivotal role in triggering disease onset. The low concordance rate observed in monozygotic twins further underlines the significance of environmental factors in triggering disease onset.

Viral Induction of Diabetes

Although direct evidence is rare, there is an abundance of circumstantial evidence linking various environmental stimuli to T1D in both human and animal models of the disease. Viruses have long been implicated as a trigger for T1D, beginning with the observations of the American clinician HF Harris in 1899 linking T1D with the mumps virus (Harris, 1899). More recent studies have shown that the mumps virus up-regulates IL-1, IL-6, HLA Class I and HLA class II expression in beta cells *in vitro*, suggesting that mumps infection may trigger β-cell destruction (Cavallo et al, 1992; Jun and Yun, 2003).

Studies performed over the past century have implicated Coxsackievirus B, Cytomegalovirus, Epstein Barr virus and Rubella infection as triggers of the disease (Szopa et al, 1993). Individuals infected with Rubella *in utero* have a 12-20% risk of developing the disease within twenty years (Ginsberg-Feller et al, 1984). It has also been shown that measles infection correlates with the presence of autoantibodies against pancreatic islet cells (Lindberg et al, 1999). However, the general use of MMP and MMR vaccines in developed countries over the past decades has essentially eliminated measles, mumps or Rubella as a likely explanation for the present "epidemic" (Lindberg et al, 1999). It has also been reported that up to 20% of T1D patients exhibit Cytomegalovirus (CMV) DNA in their pancreatic islets with many of these individuals expressing anti-CMV antibodies (Pak et al, 1988, 1990).

Studies performed with BBDR rats have shown that infection with the Kilham rat virus (KRV) induces diabetes in 30% of animals but does not influence disease incidence in BBDP rats or induce the disease in animals lacking T1D susceptibility genes (Ellerman et al, 1996; Guberski et al, 1991). Further studies have implicated both Coxsackie virus and rubella as triggers of the disease in humans although conclusive evidence is lacking (Yoon 1991,1995). In contrast, infection with lymphocytic choriomeningitis virus conferred resistance in diabetes prone BB rats (Crisa et al, 1992). Furthermore, bacterial extracts from *Streptococcus* and *E. coli* preparations have been reported to prevent the disease in diabetes-prone BB rats (Satoh et al, 1988; Kiesel et al, 1994).

Although these results show that infectious agents can influence development of disease, it remains unclear what differentiates diabetogenic and protective agents. Indeed, some people favour the "hygiene" hypothesis. It has been observed that populations plagued by infectious and parasitic diseases exhibit a much lower disease incidence than populations with more developed medical care and personal hygiene habits. This suggests that T1D protection may be associated with exposure to infectious agents during childhood (Kukreja and Maclaren, 2002).

Dietary Induction of Diabetes

Over the past two decades, research has begun to focus on the role of diet and dietary antigens in T1D. Early exposure to cow's milk has been linked to increased disease susceptibility in both human and animal models of the disease (Gerstein, 1994; Elliot and Martin, 1984; Karges et al, 1997). Furthermore, bovine β-casein variant A^1 has been associated with an increased risk of the disease in both the NOD mouse model as well as in humans (Elliot et al, 1997; Elliot et al, 1999). However, recent studies did

not support this finding but rather suggested that milk protein diets were protective in both the NOD and BB animal model (Beales et al, 2002). Vaarala and colleagues have reported enhanced proliferation of peripheral blood mononuclear cells (PBMC) isolated from T1D patients to the milk protein beta-lactoglobulin (BLG), a milk protein associated with increased disease risk. However, they failed to detect any change in proliferation to bovine serum albumin (BSA), a second cow's milk protein linked to the disease by Karjalainen and colleagues (Vaarala et al, 1996; Karjalainen et al, 1992). This group reported that antibodies reactive against the 17 amino-acid BSA peptide (ABBOS) were elevated in diabetic patients compared to controls. Furthermore, these antibodies were cross-reactive against p69, a beta-cell surface protein (Karjalainen et al, 1992). However, a subsequent study conducted by Atkinson and colleagues failed to detect any increase in ABBOS antibodies in diabetic subjects or enhanced reactivity of PBMCs to BSA (Atkinson et al, 1993). These conflicting data are representative of the disparity between studies linking milk-based diets with increased incidence of T1D in genetically susceptible individuals and other analysis which have observed no increased risk (Norris and Scott, 1996).

Dietary components of wheat gluten and soybean protein have also been implicated in increased diabetes incidence in the NOD and BBdp animal models of the disease (Scott, 1996). Diets containing L-amino acids, lactalbumin, hydrolyzed soy protein or hydrolyzed casein (HC) have been shown to inhibit the appearance of diabetes by as much as 50% in BBdp rats (Scott, 1996). Animals fed a diet containing casein as the sole source of amino acids exhibited a disease incidence of 12% compared to 63 and 65% in animals fed the diabetes-promoting cereal-based diets NIH-07 or NTP-2000

(Scott, 1996; MacFarlane et al, 2003). Furthermore, this response has been shown to be dose and time-dependent. Animals fed a 70% or 100% NIH-07 diet for 50 days or more had the highest disease frequency while animals switched to a hydrolyzed casein diet before 50 days of age were protected from the disease (Scott et al, 1997). This increase in disease frequency corresponded to a 65% reduction in islet mass and a pro-inflammatory Th1 cytokine bias in the pancreas compared to animals fed an HC diet (Scott et al, 1997).

An association has also been documented between wheat gluten and T1D in humans (Klemetti et al, 1998). PBMCs isolated from patients newly diagnosed with the disease showed increased proliferation when stimulated with wheat gluten compared to PBMCs isolated from healthy controls (Klemetti et al, 1998). Recently, antibodies against the wheat storage protein Glb-1 have been discovered in both BBdp rats and T1D patients suggesting that Glb-1 may play a role in triggering disease onset in genetically susceptible individuals (MacFarlane et al, 2003).

There is also substantial evidence linking T1D and celiac disease, a disorder characterized by abnormal immune reactivity to peptides in wheat gluten. An analysis of published reports of biopsy-proven celiac disease in T1D patients shows that T1D patients are at a 5.5% risk of developing celiac disease compared to 0.75% observed in the general population (Scott, unpublished; Fasano et al, 2003). Furthermore, the concordance rate just two decades ago was 1%. This increase appears to be associated with children suffering from T1D. Since 1995, the concordance rate in childhood sufferers of T1D has risen from 2.5% to 8% while no change was observed in adult patients suffering from T1D (Scott, unpublished).

T1D and the Gastrointestinal Link

Although T1D is linked to an inflammatory process in the pancreas, there is increasing evidence to suggest that initiation of an autoimmune response leading to β-cell destruction may originate or be facilitated in the gut. The strong link between diabetogenic dietary proteins and disease incidence in the BBdp and NOD animal models of the disease and the recent discovery of Glb-1-reactive antibodies in BBdp rats and humans suggests that diet initiates or perpetuates an autoimmune response originating from the gut of diabetes-prone subjects which then trafficks to the pancreas, targeting pancreatic β-cells and leading to onset of T1D (Scott, 1996; MacFarlane et al, 2003).

Permeability studies performed using the BBdp rat model provide further evidence of a gut-T1D link and provide a clue regarding disease progression. It has been shown that BBdp animals exhibit increased gastric and intestinal permeability by 50 days of age, corresponding to the initial stages of severe insulitis (Meddings et al, 1999). Data from our lab indicate that permeability is increased in animals as young as 40 days of age (Wang et al, submitted). Further studies have reported that both BBdp and BBc animals exhibit increased inflammatory activity in the small intestine of 70 day animals but not 45 day animals (Hardin et al, 2002). Evidence from our laboratory suggests that, similar to the pancreas, this sporadic inflammation is characterized by a Th1 response and is present in animals as young as 30 days of age. Ratios of T-bet/Gata-3, two transcription factors that control Th1 and Th2 responses were elevated in the Peyers patches (PP) and mesenteric lymph nodes (MLN) of 50 day old BBdp rats. This increase, indicative of a Th1 response, resulted solely from downregulation of Gata-3 expression. Furthermore,

IFN-γ levels were increased in the gut of these animals (Wang et al, manuscript submitted).

A similar Th1 skewed response has also been observed in the gut of NOD mice (Flohé et al, 2003). Animals fed a diabetes-promoting NTP-2000 diet expressed higher levels of the pro-inflammatory markers IFN-γ, TNF-α and inducible nitric oxide synthase (iNOS) in the small intestine than animals fed a diabetes-protective soy hydrolysate-based Prosobee diet. However, this response was not observed in the Peyers patches and was independent of any change in expression of the Th2 cytokines IL-10 or TGF-β (Flohé et al, 2003).

Although few clinical studies have been performed, preliminary data suggest that there is also significant immune activation in the jejuna of human T1D patients, leading to an inflammatory response. T1D patients were observed to have more alpha4beta7-positive ($\alpha_4\beta_7^+$) T cells in the lamina propria than controls. Alpha4beta7 is a homing receptor for mucosal vascular addressin (MAdCAM-1), a surface marker involved in the re-circulation of lymphocytes to the gut. This increase is indicative of increased inflammatory activity (Savilahti et al, 1999). Kuitunen and colleagues have also reported increased permeability to mannitol and lactulose in children with T1D expressing the HLA-DQB1*02 allele, which is associated with increased risk for celiac disease (Kuitunen et al, 2002).

From these studies, it has been hypothesized that a proinflammatory Th1 response may lead to a permeability defect and inappropriate exposure of the mucosal immune system to dietary or bacterial antigen(s) (MacFarlane et al, 2003; Hardin et al 2002). Interactions between the gut-associated immune cells and luminal antigens normally

result in tolerance towards dietary and commensal bacterial antigens. However, the gut mucosa provides only a fragile, unicellular barrier with which to regulate and minimize direct exposure of immune cells to the vast array of antigens present in the gut lumen. Thus, increased permeability could lead to overexposure of the immune system to luminal antigen, triggering a "pseudo" autoimmune response against antigen(s) to which the immune system was previously tolerized. These auto-reactive cells could then traffic to the pancreas and attack the beta cells, leading to T1D.

Although this theory remains unproven, there is increasing evidence to support such a claim. Alpha4beta7 has been observed on PBMCs isolated from T1D patients (Hanninen et al, 1998). Furthermore, MAdCAM-1 has been observed in the pancreatic infiltrates of NOD mice, suggesting that gut-derived lymphocytes may indeed traffic to the pancreas (Paronen et al, 1997).

Although the pathology of T1D differs from inflammatory bowel disease (IBD), there are some similarities which necessitate a closer look at morphological and physiological changes which occur within the gut mucosa of IBD subjects. Similar to T1D, IBD is a multi-factorial disease involving genetic predisposition, environmental factors and dysregulation of the intestinal mucosal immune response. Evidence suggests that an impaired mucosal immune response and bacterial overgrowth may lead to hyperactivation of the gut-associated immune cells towards commensal bacteria resulting in heightened inflammation in the gut mucosa (Duchmann et al, 1995; Pallone et al; 1987).

Similar to T1D, IBD involves increased intestinal permeability and inflammation, characterized by production of Th1 cytokines such as IFN-γ and TNF-α (Satsangi et al,

1996; MacDonald et at, 1994; Rhodes, 1996). This Th1 predominance is associated with increased permeability and overexposure to bacterial antigens residing in the gut lumen, resulting in gut inflammation and damage (Rhodes, 1996).

The Use of Interleukin-10 In the Treatment of T1D

The destructive nature of the Th1 response observed in both T1D and IBD has recently become a target for the treatment of each of these diseases. Of particular interest is the use of the anti-inflammatory Th2 protein IL-10, a potent immunosuppressive cytokine implicated in protection against T1D and IBD (Ding et al, 2000; Syto et al, 1998).

IL-10 is a 178 amino acid homodimer encoding an 18.45 kDa protein in the mouse and rat and an 18.65 kDa protein in humans (Syto et al, 1998; Ball et al, 2001). All three forms exhibit 6 alpha helices with structural integrity provided by 2 disulphide bonds (Ding et al, 2000; Windsor et al, 1993). Reduction of these bonds leads to the formation of an inactive monomer below pH 5.5 although a loss of biological activity may be observed as high as pH 6.5 (Schotte, 2000). Studies have shown that biological activity is directly proportional to the percent homodimer (Syto et al, 1998).

IL-10 is highly conserved with a structural homology of 73% at the amino acid level between murine and human IL-10 and 83% between mouse and rat IL-10 (Ball et al, 2001). The Epstein-Barr virus BCRF-1 gene product, known as viral IL-10 (vIL-10) is also structurally similar to mammalian forms of IL-10 and has proven useful in the treatment of both IBD and T1D (Ding et al, 2000; Goudy et al, 2002; Yang et al, 2002).

The immunosuppressive capabilities of IL-10 are numerous and include the down-regulation of the pro-inflammatory cytokines IL-1α/β, IL-2, IL-3, IL-6, IL-8, IL-

12, IFN-γ, TNF-α, TNF-β and GM-CSF (Howard et al, 1992). Other immunosuppressive roles include down-regulation of MHC Class II expression, co-stimulatory B7/1 and B7/2 expression and decreased production of IgE antibodies (Muller et al, 1998; de Waal Malefyt et al, 1991; Punnonen, et al, 1993).

In studies performed with the NOD mouse model of T1D, intramuscular treatment with recombinant hIL-10 (human IL-10) beginning at 70 days prevented the disease in the majority of recipients (Pennline et al, 1994). Further datum suggest that although earlier treatment does not enhance this effect, delay of treatment until 90 days greatly diminished its effectiveness (Pennline et al. 1994). As complete β-cell destruction does not occur until approximately 85 days of age in the NOD model, early treatment may halt initial stages of β-cell destruction whereas with later treatment, this damage has already been incurred. Molecular analysis from this study suggests that although recombinant hIL-10 does not prevent insulitis, it does down-regulate MHC Class II expression on monocytes, leading to suppressed T cell activity and down-regulation of proinflammatory cytokines (Pennline et al, 1994). Because hIL-10 inhibited disease appearance but not insulitis, this suggests that IL-10 treatment resulted in a benign, Th2-based form of insulitis.

In two other studies, vIL-10 was introduced via intra-muscular delivery of the Adeno-Associated virus (AAV) vector. Once again, MHC Class II expression was diminished on antigen presenting monocytes leading to decreased T cell proliferation and a reduction in synthesis of the IFN-γ and IL-2 by $CD4^+$ T lymphocytes. Serum autoantibody titers and insulitis were also low (Yang et al, 2002; Goudy et al, 2002).

The association between a Th1 skewed response and IBD is also well documented through the use of the IL-$10^{-/-}$ mouse model which spontaneously develops IBD. The absence of IL-10 in this model has been shown to impair the $CD4^+$ T_{reg} response, leading to increased production of the proinflammatory Th1 cytokines IFN-γ and IL-12 (Powrie et al, 1994). Furthermore, intramuscular delivery of exogenous IL-10 has been shown to reduce disease incidence and severity in dextran sodium sulphate- (DSS) induced and $CD4^+CD45RB^{high}$ models of colitis (Tomoyose et al, 1998; Powrie et al, 1994). Intramuscular delivery of hIL-10 has also been shown to attenuate IBD in 50% of patients compared to 23% observed in placebo-treated patients. This amelioration corresponds with down-regulation of proinflammatory cytokine release from lamina propria mononuclear cells (Van Deventer et al, 1997; Schreiber et al, 1995).

Because the Th1-skewed cytokine bias observed in IBD is localized within the gut, it has been suggested that the efficacy of IL-10 treatment may be enhanced if delivered directly to the target tissue. However, this provides a unique challenge given that oral delivery of IL-10 is complicated due to the acidic nature of the stomach and proximal duodenum (Schotte et al, 2001). An additional concern is the fact that mIL-10 is very acid labile and is completely inactivated at pH5.5; the pH of stomach acid is 1.5 (Syto et al, 1998).

The recent development of Lactococcus lactis pTRmIL-10 (pTRmIL-10), genetically engineered to secrete biologically active mIL-10, is a significant breakthrough in the development of functional probiotics, which are live microbial food ingredients capable of altering the enteric flora and conferring a health benefit (Naidu et al, 1999). This gram-positive bacteria is a member of the non-pathogenic, acid resistant Lactic Acid

Bacteria (LAB) family. These properties allow *L. lactis* pTRmIL-10 to survive passage through the harsh environment of the stomach and proximal duodenum and produce biologically-active mIL-10 in the more moderate conditions of the distal gut lumen and colon, allowing delivery directly to the target tissue.

In initial studies using this novel vector, pTRmIL-10 was used to treat mice suffering from dextran sulfate sodium (DSS)-induced IBD. Chronic colitis was induced in mice by the administration of 5% DSS for 7 days followed by a 10 day recovery period. After repeating this routine 4 times, animals were allowed final recovery period of 21 days before treatment with 2×10^7 CFU of pTRmIL-10 was initiated. 50% of animals treated with pTRmIL-10 for two weeks had a significant reduction in gut inflammation as determined by blind histological assessment. Although delivery of pTRmIL-10 for a longer period of time did not confer any added benefit, delivery of 2×10^9 CFU per day proved less effective in attenuating the disease (Steidler et al, 2000).

Steidler and colleagues also tested the use of pTRmIL-10 in the treatment of spontaneous IBD in IL-$10^{-/-}$ mice which develop the disease within 8 weeks of life. Oral delivery of a total of 2.4×10^{10} CFU of pTRmIL-10 initiated at 3 weeks of age prevented the disease in all animals (Steidler et al, 2000; Berger, 2000). Furthermore, this prevention was directly attributed to pTRmIL-10 as treatment with heat-killed pTRmIL-10 did not prevent disease onset (Steidler et al, 2000).

Given the success of *L. lactis* pTRmIL-10 treatment in the attenuation and prevention of experimentally induced colitis, the striking similarity in the Th1-cytokine profile observed in the gut of IBD and T1D subjects and the successful use of vIL-10 and hIL-10 in the attenuation of T1D in the NOD mouse, we propose that the *in vivo*

production of mIL-10 produced by *L. lactis* pTRmIL-10 will counter-balance the Th1-cytokine bias observed in the gut of diabetes-prone BB rats and inhibit development of T1D.

Hypothesis

T1D is a multifactorial disease triggered by the presence of various risk factors. These include, but are not exclusive to genetic risk factors, viral infection and dietary constituents. The latter observation, along with the possible role of enterovirus infection in triggering disease onset has led to considerable interest in elucidating the role of the gut in the etiology of T1D. A few reports have associated T1D with increased intestinal permeability, inflammation and trafficking of gut-derived lymphocytes to the pancreas.

There is mounting evidence to suggest that the proinflammatory response observed in the gut of T1D subjects is associated with an abnormal Th1-skewed cytokine bias, similar to the cytokine profile documented in the pancreas of diabetes-prone and diabetic subjects. It has been proposed that this bias may be a contributing factor in the initiation of T1D and that delivery of a Th2 cytokine may neutralize this inflammatory process and inhibit the disease.

Preliminary studies involving intramuscular and AAV-delivery of the Th2 cytokine IL-10 to the NOD mouse support this claim. However, this method of delivery does not allow IL-10 to be directed towards the intestinal mucosa. Therefore, we propose that oral delivery of the mIL-10 secreting bacteria *L. lactis* pTRmIL-10 will suppress the proinflammatory Th1 response observed in the gut of BBdp rats and inhibit development of the disease.

Research Goals

1. To quantify the production of mIL-10 by *L. lactis* pTRmIL-10 *in vitro* and evaluate the biological activity of the mIL-10.

2. To determine if *L. lactis* pTRmIL-10 survives transit through the stomach and proximal duodenum of BBdp rats and to quantify pTRmIL-10 levels in the stomach, duodenum, ileum, jejunum and colon.

3. To determine if oral delivery of *L. lactis* pTRmIL-10 is associated with an increase in mRNA expression of Th2 cytokines and/or a decrease in the expression of Th1 cytokines in the proximal duodenum of BBdp rats.

4. To determine if oral delivery of *L. lactis* pTRmIL-10 is associated with the prevention of T1D in BBdp rats.

Materials and Methods

Characterization of L. lactis pTRmIL-10 and pTR1NX

Bacterial Culture Protocol for L. lactis

Lactococcus lactis control strain p663 (p663), control plasmid strain pTR1NX (pTR1NX) and the murine IL-10-producing strain pTRmIL-10 (pTRmIL-10) were generously provided by Dr. L Steidler (University of Ghent, Ghent, Belgium). All strains were routinely cultured overnight in M17 growth medium containing 0.5% glucose (Difco; Appendix 1), a medium specially formulated for the growth of lactic streptococci. PTR1NX and pTRmIL-10 cultures were supplemented with 5 μg/mL of erythromycin (Sigma). All incubations were performed at 30°C with gentle agitation in a rotary incubator.

Upon saturation (A_{600} ~1.7), bacteria were diluted 1:100 into fresh M17 medium and cultured for 3 hours prior to re-suspension (10 min @ 2000 × g) in bicarbonate-buffered M9 Minimal Salts medium (BM9), pH 8.5, a refined medium suitable for the isolation of soluble protein. BM9 was prepared by supplementing M9 (Difco; Appendix 2) with 5 g of casitone peptone (Difco) per litre, 50 mM of sodium bicarbonate and 0.5% glucose. PTR1NX and pTRmIL-10 cultures were additionally supplemented with 5 μg/mL of erythromycin. Cultures were harvested 5 hours post-inoculation (10 min @ 2000 × g) and the supernatants collected.

In Vitro mIL-10 Quantification

To quantify mIL-10 production by pTRmIL-10 five hours post-inoculation in BM9, mIL-10 levels were measured using a commercially available mIL-10 ELISA kit

(BD Pharmingen). Plates were prepared according to the manufacturer's protocol. Samples were serially diluted in fresh BM9 and assayed in triplicate. Absorbance values were determined at 450 nm wavelength and mIL-10 concentrations by comparison with a standard curve (BioRad).

PTRmIL-10 and PTR1NX Protein Isolation from BM9

To aid in the characterization of protein produced by each construct, protein was collected from pTR1NX and pTRmIL-10 supernatants 5 hours post-inoculation in BM9. Briefly, protein was extracted from the supernatant using a half volume of phenol (10 min @ 10,000 × g) and precipitated overnight by the addition of 2.5 × volume of ethanol. Protein pellets (10 min @ 2000 × g) were subsequently washed in water-equilibrated diethyl ether, prepared by mixing an equal volume of distilled water and diethyl ether and using the top phase. Pellets were re-suspended in a Cracking Buffer, protein denaturing solution consisting of 8 M urea, 5% w/v SDS, 40 mM Tris-HCl, 0.1 mM EDTA and 0.4 mg/mL of Bromophenol blue; pH 6.8. Total protein was quantified using the Bradford assay (BioRad; Bradford, 1976).

SDS-PAGE of pTRmIL-10 and pTR1NX Protein Isolate

To compare proteins produced by the pTR1NX and pTRmIL-10 plasmid constructs, protein isolates were separated on 15% SDS polyacrylamide gels (150V, 109 mAmp) and visualized using silver nitrate staining. Briefly, gels were fixed in a 50% EtOH/ 5% acetic acid solution (v/v) for 30 minutes followed by sensitization in a 0.02% sodium thiosulphate solution (w/v) for 2 minutes. Protein was stained for 30 minutes in a 0.1% silver nitrate solution (w/v) and developed in a 0.04% formalin/2% sodium

carbonate solution (w/v). Development was stopped at the appropriate time using a 5% acetic acid solution (v/v).

Western Blot Analysis of pTRmIL-10 and pTR1NX Protein Isolate

To confirm the production of mIL-10 by pTRmIL-10, protein isolates from pTR1NX and pTRmIL-10 were separated on 15% SDS polyacrylamide gels (150V, 109 mAmp, ~1.5 hours) and electrophoretically transferred onto nitrocellulose membranes (100 V, 250 mAmp, ~1.5 hours). Samples were probed with monoclonal rat anti-murine IL-10 antibody (R&D Systems, 1:1500) and labeled using an alkaline phosphatase-conjugated goat anti-rat IgG antibody (Jackson Laboratories, 1:1500).

Confirmation of mIL-10 Biological Activity using the MC/9 Bioassay

To confirm that mIL-10 secreted by *L. lactis* pTRmIL-10 was biologically active, the IL-4/IL-10-sensitive MC/9 murine mast cell line (ATCC) was cultured in Dulbecco's Modified Eagle Medium (DMEM)(Sigma) supplemented with 0.05 mM of β-mercaptoethanol (Sigma), 10% fetal bovine serum (FBS; Sigma) and conditioned with 5% T-Stim Culture Supplement (BD Pharmingen). Cells were cultured for a minimum of one week prior to performing the bioassay. Because filtration may denature and inactivate mIL-10, 5 hour BM9 cultures were subjected to multiple high-speed centrifugations (3 × 10 min @ 10,000 × g) to remove residual bacteria and frozen at –20 °C until further use.

For the MC/9 bioassay, cells were seeded at a concentration of 1×10^4 cells/mL in unconditioned DMEM and cultured in the presence of mIL-4 and mIL-10 (Peprotech) or mIL-4 and protein isolated from pTRmIL-10 for 72 hours. Cells were mixed with trypan

blue (Sigma) to determine viability and counted in a hemocytometer to distinguish live and dead cells (Appendix 3).

Oral Treatment of BBdp rats with *L. Lactis* PTRmIL-10

Preparation of L. lactis for gavage

In preparation for the oral treatment of BBdp rats with *L. lactis* pTRmIL-10, *L. lactis* p663, pTR1NX, and pTRmIL-10 were grown in the appropriate M17 growth medium until saturation (A600 = 1.7; 2×10^9 CFU/mL). Bacterial pellets (10 min @ 2000 × g) were re-suspended in an equal volume of M17 Freeze Medium (1:1 v/v of M17/Glycerol) to a final concentration of ~2×10^9 CFU/mL.

For quality control, bacterial concentrations were quantified using standard plate count techniques (Association of Official Analytical Chemists, 1990). Dilutions ranging from 1×10^5 to 1×10^8 were plated on M17 agar plates and incubated for 48 hours. M17 agar was prepared by the addition of 15 g of bacterial agar (Difco) per litre of M17 and autoclaved prior to the addition of 0.5% glucose. 5 μg/mL of erythromycin was added to pTR1NX and pTRmIL-10-selective plates. Plates containing <30 and >300 colonies were discarded as per standard plate count protocol (Tomasiewicz et al, 1980). Final counts were back-calculated to confirm the concentration of the inoculum. Formulations were subsequently stored at –20°C until further use. Viability was assessed weekly thereafter using standard plate count procedures as described above.

Animals

160 weanling BBdp rats were obtained from the Animal Resources Division of Health Canada, Ottawa, Canada. Animals were randomly chosen and maintained individually in stainless steel wire-bottom cages. Animals were routinely tested and

found to be free of antibodies against Sendai virus, pneumonia virus of mice, rat coronavirus, rat sialodacryoadenitis virus, KRV, Toolan's H-1 virus, reovirus type 3 and *Mycoplasma pulmonis*. Historically, diabetes incidence is 65.3 ± 14.9 % (n= 6 experiments, total of 169 rats) with age at onset ranging from 55-130 days of age.

Diet

Animals were weaned onto a diabetes-promoting NTP-2000 diet at approximately 23 days of age and permitted free access to food and water throughout the course of the study. The NTP-2000 diet consists of (w/w) 22.18% ground corn, 22.26% ground wheat, 15% wheat middlings, 5% soybean meal, 4% fish meal, 7.5% alfalfa meal, 8.5% oat hulls, 5.5% purified cellulose, 3% corn oil, 3% soy oil, 1% dried brewers yeast, 0.3% sodium chloride, 0.4% calcium phosphate dibasic, 0.9% calcium carbonate, 0.26% choline chloride and 0.5% respectively of vitamin and mineral premix.

Oral Administration of L. lactis pTRmIL-10 and Controls

Animals were randomly divided into four groups of 40 and gavaged daily with one of the following treatments; M17 Freeze medium control, *L lactis* p663, *L lactis* pTR1NX or *L lactis* pTRmIL-10. Gavaging was initiated at approximately 26 days of age with a daily delivery of 1 mL containing 2×10^9 CFU. Treatment was continued until diagnosis of diabetes or 160 days of age. Subgroups were sacrificed at 45 (n=7) and 70 days (n=8) of age for prospective analysis.

Diabetes Incidence in BBdp Rats Treated with pTRmIL-10

Animals were tested twice weekly for the presence of urinary glucose using Testape strips (Lilly) beginning at ~50 days of age. Animals with a urinary glucose level of +2 or higher were fasted overnight and blood glucose levels were measured the

following morning using a glucometer. Animals with a blood glucose of 11.1 mM or higher were diagnosed as diabetic and sacrificed within 24 hours. All animals were killed by exsanguination under 3% halothane in oxygen anaesthesia.

Tissue Collection

Blood was collected from the dorsal aorta of anaesthesitized animals into SST Gel and Clot Activator tubes (BD Pharmingen). Serum was separated by centrifugation (10 min @ 2,000 × g) and frozen at –20 °C until further use.

Isolation of Duodenal RNA

Two centimeters of proximal duodenum located approximately 6 cm from the pylorus was aseptically excised from anaesthesitized animals and washed in phosphate-buffered saline (PBS; 37 °C) prior to being snap-frozen in liquid nitrogen. RNA isolation was performed subsequently. Briefly, samples were homogenized in 3 mL of Trizol reagent (Invitrogen) and total RNA was isolated according to the protocol recommended by the manufacturer. Isolated RNA was then purified using two consecutive 70% ethanol washes prior to re-suspension in DEPC-treated water. Dithiothreitol (DTT; Sigma) and RNasin (Promega) were added at a concentration of 0.0025 M and 0.4U/μL respectively. Samples were frozen at –80 °C until further use.

In Vivo Quantification of L. lactis pTR1NX and pTRmIL-10

To quantify pTR1NX and pTRmIL-10 levels in the gut, standard plate counts were performed on the digestive material (Association of Official Analytical Chemists, 1990). Briefly, digesta and fecal material were collected from the stomach, duodenum, jejunum, ileum, and colon of pTR1NX and pTRmIL-10-treated animals. After weighing, the contents were diluted 10 times w/v in M17 and vortexed to separate bacteria from

undigested material. The resulting slurry was diluted 1×10^3 to 1×10^8 times and plated on M17 agar containing 5 μg/mL of erythromycin. Plates were incubated for 48 hours at 30 °C. Only plates containing 30 to 300 colonies were counted. Final counts were then back-calculated to determine the CFUs/g of fecal or digesta content (Appendix 4). Statistical comparisons were performed using one-way ANOVA (LSD post-hoc analysis; if $p \leq 0.05$, means were considered to be significantly different).

Serum Dot-Blot Analysis

To determine whether animals developed reactivity against mIL-10, 2.5-5 μg of commercial mIL-10, ratIL-10 and pTRmIL-10 protein extracts were blotted onto nitrocellulose membranes and blocked overnight in 5% skim milk. Blots were probed with commercial monoclonal rat anti-mIL-10 (R&D, 1:1,500), pooled serum from animals treated with pTRmIL-10 (1:50) or pooled serum pre-absorbed with 40 to 80 ng/mL of commercial mIL-10 (Peprotech; 1:50). Individual sera collected from animals treated with pTRmIL-10 were also used to probe (1:50) the above proteins as well as *E. coli* and *L. lactis* lysate.

RT-PCR of IFN-γ, IL-10 and IL-4 Expression in the Duodenum

Analysis of gene expression of representative Th1 (IFN-γ) or Th2 (IL-4 and IL-10) cytokines was performed to determine the impact of pTRmIL-10 on the Th1/Th2 balance in the gut of BBdp and BBd animals. Reverse transcription was performed with 2 μg of total duodenal RNA using an oligo $(deoxythymidine)_{16}$ primer and a GeneAmp RNA PCR kit (Roche/Applied Biosystems). PCR was performed using the GeneAmp PCR kit. IL-10, IFN-γ, and IL-4 primers were designed according to the corresponding rat gene (Appendix 5). PCR annealing temperatures were; IL-10: 55 °C, IFN-γ: 53 °C,

IL-4: 63 °C and β-actin: 55 °C. PCR cycle numbers were optimized for each experimental condition and primer set. Representative samples were run at different cycle numbers and the optimal cycle number was selected in the region of linearity between cycle number and PCR product intensity. The absence of PCR product signal from genomic DNA contamination was confirmed by performing PCR with representative RNA samples without reverse transcription amplification of mRNA. Optimized cycle numbers were; β-actin: 23 cycles; IL-10, IFN-γ and IL-4: 37 cycles. PCR product sizes were 461 (IFN-γ), 346 (IL-10), 420 (IL-4) and 343 (β-actin).

Semi-quantitative RT-PCR was performed in duplicate normalizing against β-actin as an internal control. PCR products were electrophoresed in 1.5% agarose gel and the net band intensity was analysed using a Kodak image station 440CF (Eastman Kodak, Rochester, NY). Statistical comparisons were performed using one-way ANOVA (LSD post-hoc analysis; if $p \leq 0.05$, means were considered to be significantly different).

In Vitro mIL-10 and rat IL-10 Quantification

Individual sera collected from animals treated with pTRmIL-10 (n =10) were screened for the presence of mIL-10 using a commercially available mIL-10 ELISA kit (BD Pharmingen). Representative sera from each study group (Buffer, p663, pTR1NX , pTRmIL-10; n = 8/group) were also screened for the presence of rat IL-10 using a commercially available rat IL-10 ELISA kit (BioSource International). Plates were prepared according to the manufacturer's protocol and a serial dilution (1:2, 1:10, 1:100) of each sample was prepared. Samples were assayed in triplicate and were quantified

against the standard provided with the kit. ELISA sensitivity was reported to be 15 pg/mL when standards are replicated ≥20 times.

Results

In Vitro mIL-10 Quantification

To confirm production of mIL-10 by pTRmIL-10 *in vitro*, mIL-10 levels were assayed using a commercially available ELISA kit. Results indicate that 5 hours post-inoculation in BM9, L. lactis pTRmIL-10 supernatants contained 485 +/-33 ng/mL of mIL-10 (n = 3).

SDS PAGE of pTRmIL-10 and pTR1NX Protein Isolate

To compare proteins secreted by pTR1NX and pTRmIL-10, SDS PAGE was performed on 5 hour BM9 cultures of pTRmIL-10 and pTR1NX. Results indicate that a protein band corresponding to mIL-10 (18.45 kDa) was present in pTRmIL-10 cultures but not in pTR1NX cultures (Fig 1).

Western Blot Analysis of pTRmIL-10 and pTR1NX Protein Isolate

To confirm production of mIL-10 by pTRmIL-10, Western blot analysis was performed on pTR1NX and pTRmIL-10 protein isolates. Results indicate the presence of mIL-10 (18.45 kDa) in pTRmIL-10 cultures but not in pTR1NX cultures (Fig 2).

MC/9 Bioassay of the Biological Activity of mIL-10 produced by pTRmIL-10

To ascertain whether mIL-10 produced by pTRmIL-10 was biologically active, MC/9 cells were co-incubated with pTRmIL-10 supernatant and mIL-4. Results indicate ~50% increase in proliferation when using 400 pg/mL of IL-4 and 2.43 ng/mL of mIL-10 as determined by ELISA (Fig 3). Bars sharing the same letter denote statistically significant differences ($p \leq 0.05$) as determined by ANOVA followed by Fisher LSD post-hoc analysis (Fig 3).

Quantification of L. lactis pTR1NX and pTRmIL-10 In Vivo

To determine the efficacy of *L. lactis* pT1NX and pTRmIL-10 transit and survival in the gastrointestinal tract, standard plate counts were performed on the contents of the stomach, duodenum, jejunum, ileum and colon. 10^4 to 10^5 bacteria per gram of digesta were observed in the stomach of pTR1NX and pTRmIL-10 treated animals respectively. These numbers increased in the ileum and jejunum to 2×10^5-1×10^8 in colon of pTR1NX-treated animals and 1×10^6-1×10^8 in animals treated with pTRmIL-10. Both pTR1NX and pTRmIL-10 levels were increased in the duodenum, ileum, jejunum and colon of diabetic animals compared to 45 day, 70 day and end-of study animals. pTRmIL-10 was also elevated in the stomach of diabetic animals compared to 45 day, 70 day and end-of-study animals but pTR1NX was absent in the corresponding control group (Fig 4 & 5). Letters denote statistically significant differences between the corresponding bars ($p \leq 0.05$) as determined by ANOVA (Fisher LSD post-hoc analysis).

Diabetes Outcome

Oral delivery of *L. lactis* pTRmIL-10 was associated with increased T1D incidence in the BBdp rat (86.4%) compared to animals treated with *L. lactis* p663 and pTR1NX (68%). This difference was not statistically significant (pTRmIL-10 vs p663 and pTR1NX, $p = 0.6606$), indicating no effect of pTRmIL-10 on diabetes incidence. Oral treatment with M17 Freeze Buffer was associated with a disease incidence to 44% (Buffer vs pTRmIL-10, $p = 0.0054$; Buffer vs p663 and pTR1NX, $p = 0.4783$). Statistical significance was determined by the Fisher Exact test comparing final diabetes frequency (Fig 6).

Serum Dot Blot analysis

Although the incidence seen in pTRmIL-10-treated BBdp rats was not significantly different from bacterial controls, this incidence was among the highest ever recorded in rats from the Ottawa colony. Therefore, it was decided to investigate the possibility that reactivity against mIL-10 may be implicated. To test this, pooled serum collected from animals treated with pTRmIL-10 was used to probe commercial mIL-10, rat IL-10 and pTRmIL-10 protein isolate. Results showed that antibodies from these animals were reactive against pTRmIL-10 protein isolate but no reactivity was observed against purified mouse or rat IL-10 (Fig 7). Pooled sera pre-absorbed with 40-80 ng/mL of commercial mIL-10 showed similar reactivity against pTRmIL-10 protein isolate but not against mouse or rat IL-10 (Fig 8).

RT-PCR Analysis of Duodenal RNA for Th1 and Th2 Cytokines

Expression of IFN-γ, IL-10 and IL-4 genes was analyzed to ascertain the impact of oral delivery of pTRmIL-10 on the Th1/Th2 cytokine balance in the gut. RT-PCR analysis of duodenal tissue showed increased IL-10 expression in 70 day animals treated with buffer alone compared to 45 day, diabetic and end of study animals (Fig 9; $p \leq 0.05$). No difference was observed in IL-10 expression in the duodenum of animals treated with p663, pTR1NX or pTRmIL-10 (Fig 9).

IFN-γ expression was increased in the proximal duodenum of diabetic animals treated with buffer alone as well as in end-of study animals treated with p663. Intra-group analysis shows an increase in the expression of IFN-γ in diabetic animals treated with buffer alone compared to 45 day, 70 day and end of study animals (Fig 10).

IL-4 expression was increased in the proximal duodenum of 45 day old animals treated with p663 compared to those treated with pTR1NX and pTRmIL-10 but not in animals treated with buffer alone. Diabetic animals treated with buffer alone also exhibited increased IL-4 expression compared to animals treated with pTR1NX and pTRmIL-10 but not those treated with p663. Intra-group analysis shows that IL-4 expression was increased in 45 day animals treated with p663 compared to 70 day, diabetic and end-of-study animals. No changes were observed in animals treated with buffer, pTR1NX or pTRmIL-10 (Fig 11).

In Vitro mIL-10 and rat IL-10 Quantification

To determine if mIL-10 entered the circulatory system of animals treated with pTRmIL-10, commercial mIL-10 and rat IL-10 ELISAs were performed on sera. mIL-10 was undetectable in individual sera collected from animals treated with pTRmIL-10. Furthermore, rat IL-10 was undetectable in representative serum samples collected from all groups (buffer, p663, pTR1NX, and pTRmIL-10).

Discussion

It has been recognized for some time that T1D in the BBdp rat is associated with diabetes-promoting dietary constituents (Scott, 1996). Diets with a high wheat protein content have been shown to promote the development of diabetes while semipurified diets containing hydrolyzed casein as the sole amino acid source have been shown to be diabetes protective. Recently, the wheat protein Glb-1 has been implicated as one dietary antigen which may induce the disease. Antibodies against this peptide are present in the serum of diabetic animals treated with diabetes-promoting NTP-2000 and NIH-07 diets with a close association observed with islet inflammation and damage (MacFarlane et al, 2003).

There is also evidence linking T1D in the BBdp rat to increased intestinal permeability. In a study performed in our lab, permeability to mannitol, a measure of paracellular permeability was higher in 41 day BBdp animals compared to control animals (Wang et al, Submitted). Hardin and colleagues have also observed that myeloperoxidase (MPO), a marker of increased intestinal permeability, is increased in 45-day-old BBdp rats and in 70 day BBdp and BBd animals (Hardin et al, 2002; Meddings et al, 1999). These early time-points correspond to the initial stages of pancreatic insulitis, that usually starts around 50-55 days (Guberski, 1994).

Recently, research from our lab has suggested that this gut dysfunction is linked to a Th1 cytokine bias in the gut leading to inflammation. T-box expressed in T cells (T-bet) and GATA-binding protein-3 (GATA-3), recently identified as indicators of a Th1 and Th2 response, were measured in the gut of pre-insulitic BBdp animals. Although no change was observed in T-bet levels, GATA-3 expression was decreased in the

mesenteric lymph nodes (MLN) and Peyer's Patches (PP) compared to controls, indicating a shift towards a Th1-skewed response (Wang et al, submitted).

L. lactis as a Delivery Tool for mIL-10

As mentioned previously, recombinant IL-10 has been successfully used in the treatment of both experimentally-induced IBD as well as T1D in the NOD mouse model. Prior to the development of *L. lactis* pTRmIL-10, IL-10 delivery was primarily through intra-muscular (IM) injections. In studies performed with NOD mice, IM delivery of hIL-10 prevented T1D in the majority of recipients (Pennline et al, 1994). Furthermore, IM delivery of IL-10 reduced disease incidence in DSS and $CD4^{+}CD45RB^{high}$- induced models of colitis. IM delivery of IL-10 to IBD patients has also proven useful in attenuating disease severity in 50% of recipients. Although clinical studies have demonstrated that daily systemic injections are safe and well-tolerated, two studies have shown that this form of delivery has minimal therapeutic efficacy on induced IBD models when compared to placebo (Fedorak et al, 2000; Schreiber et al, 2000).

A second, more novel delivery mechanism is the development of IL-10-encoding Adeno-viral (AV) and Adeno-associated viral (AAV) vectors. In two studies, vIL-10 introduced via intra-muscular delivery of the Adeno-Associated virus (AAV) vector reduced diabetes incidence in the NOD mouse model (Yang et al, 2002; Goudy et al, 2001). In a more recent study, Lindsay and colleagues have shown that rectal delivery of an mIL-10-encoding adenoviral vector attenuates inflammatory colitis in an $IL\text{-}10^{-/-}$ model of IBD (Lindsay et al, 2003).

Although AV and AAV delivery of IL-10 has proven effective, an important shortcoming is that control over the duration and quantity of IL-10 delivery is lost.

Lindsay and colleagues have demonstrated that a single systemic delivery of an IL-10-encoding adenoviral vector results in the production of biologically active IL-10 for a minimum of 10 weeks. This long-term delivery leads to impaired splenocyte response to lipopolysaccharide (LPS) as well as a dose dependent anemia and thrombocytopenia (Fedorak et al, 2000; Schreiber et al, 2000).

Because of the association between IBD and a Th1-skewed cytokine response in the gut, it has been hypothesized that the efficacy of IL-10 treatment in IBD may be enhanced by direct delivery to the diseased site. This provides a formidable challenge, as the task of oral delivery of IL-10 is complicated because of the acidic environment encountered in the stomach (pH ~1.5) and proximal duodenum. A loss of IL-10 biological activity is observed at pH ~6.5 and the protein is completely inactivated at pH 5.5.

The use of recombinant bacteria for production and oral delivery of recombinant proteins and antigens has been well documented over the past decade (Wells et al, 1996). The primary focus has been directed towards the use of live, attenuated strains of pathogenic genera such as *Mycobacterium*, *Salmonella*, *Clostridia* and *E. coli*. The effectiveness of these vectors is believed to depend in part to their invasiveness and capacity to survive. However, it is these attributes which make attenuated pathogens potential candidates for opportunistic infections in pediatric, geriatric and immuno-compromised patients. Furthermore, it remains unclear what impact prolonged exposure to large dosages of these recombinant pathogens will have on healthy individuals.

The use of recombinant bacteria for the production of IL-10 is also well documented (Ball et al, 2001). *E. coli* has been utilized for some time in the production

of IL-10, although its use has been limited primarily to *in vitro* studies. Although *E. coli* is a potential opportunistic pathogen, a more serious limitation is the packaging of IL-10 in inclusion granules as it is secreted from the bacteria. Protein isolation from these granules can only be achieved after a complex, labour-intensive solubilisation and renaturation process (Ball et al, 2001).

More recently, lactic acid bacteria (LAB) have been investigated as potential vehicles for oral delivery of various antigens and recombinant proteins. Members of this family are Gram positive, non-pathogenic, non-invasive bacteria. Many genera, such as *Lactobacillus*, *Bifidobacterium* and *Lactococcus spp* are used in the fermentation of food products including cheese, yogurt, and sauerkraut (Naidu et al, 1999). These food-grade bacteria have also been granted Generally Regarded as Safe (GRAS) status by the US Food and Drug Administration (Naidu et al, 1999).

Most genera of LAB are also characterized as "probiotics". Probiotics are defined as "microbial dietary adjuvants that beneficially affect the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract"(Naidu et al, 1999). As the definition implies, these bacteria mediate a delicate balance that exists between the resident microflora and the intestinal mucosal immune response of the host. This role is achieved through a broad range of biochemical, physiological and immuno-modulatory functions (Naidu et al, 1999).

Oral treatment with *Lactobacillus casei* GG has been shown to reverse intestinal permeability in rats, induced by oral treatment with bovine milk (Isolauri et al, 1993). This same strain has also been shown to decrease microflora-induced proliferation of peripheral $CD4^+$ T lymphocytes and is associated with increased IL-10 production and

decreased IFN-γ and TNF-α production (Schultz et al, 2002). Oral treatment with heat-killed *Lactobacillus casei* has also been shown to reduce diabetes incidence in the NOD mouse model (Matsuzaki et al, 1997). Animals fed a cereal-based diet exhibited a diabetes incidence of 83.5% while animals fed diet supplemented with 0.05% w/w of *L. casei* had a disease incidence of only 16.5%. This supplement was initiated at 4 weeks of age and was continued throughout the course of the study (Matsuzaki et al, 1997). Given the attributes of probiotics in maintaining a balanced normal flora, their GRAS status and their acid-resistant nature, their utilization as an oral delivery vehicle may prove to be a valuable breakthrough in the safe and effective delivery of protein to the gut lumen.

The use of *Lactococcus lactis* for the development of an mIL-10-producing vector had one overriding quality over other probiotic species. As well as exhibiting all of the characteristics which make other LAB genera useful for oral delivery of antigen or recombinant protein, *L. lactis* is a non-colonizing member of the allochthonous, or transient flora. Studies have shown that *L. lactis* cells transit the gut within 3 days of oral delivery (Wells et al, 1996). This provides an effective means of control over the quantity and duration of delivery unlike colonizing LAB which may produce recombinant protein for an extended period of time.

Use of mIL-10 in the Treatment of T1D in the BBdp rat

The successful treatment of induced murine colitis by Steidler *et al* provided evidence that oral delivery of *L. lactis* pTRmIL-10 produced biologically active mIL-10 in the intestinal tract of diseased animals and prevented IBD by altering gut inflammation. What was unclear was whether murine IL-10 would exert biological activity and prevent T1D onset in the BBdp rat. Previous studies have shown that

delivery of hIL-10 and vIL-10 are capable of preventing T1D in the NOD mouse, suggesting inter-species activity. Homology between hIL-10 and mIL-10 is 73% whereas homology between mIL-10 and rIL-10 is ~ 83%. Furthermore, it is well documented that rat, human and mIL-10 are all capable of inducing proliferation in the IL-4/IL-10-dependent MC/9 murine mast cell line. These results provided circumstantial evidence that mIL-10 may be biologically active in other murine models and prompted the present attempt to prevent T1D in the BBdp rat.

In Vitro Production of mIL-10 by L. lactis pTRmIL-10

The quantity of mIL-10 in pTRmIL-10 cultures was determined through the use of SDS PAGE and immunoblotting techniques as well as by ELISA. A band was observed at the 18.5 kDa region, corresponding to the molecular mass of mIL-10 (Fig 1 & 2). Image analysis showed that IL-10 accounts for approximately 25% of the total protein secreted by pTRmIL-10 (Appendix 6), corresponding with the results of Schotte *et al* which indicate 30% of total protein secreted by pTRmIL-10 is mIL-10 (Schotte et al, 2000). Quantification of mIL-10 production showed that 485 ng/mL was produced 5 hours after pTRmIL-10 inoculation in BM9 medium.

A major byproduct of all LAB is lactic acid, which can rapidly decrease the pH of *L. lactis* cultures to 5.5 providing the nutrient is sufficient. Given the acid-sensitivity of mIL-10, culturing *L. lactis* in traditional M17 growth medium (pH 6.5-7) would quickly lead to inactivation and degredation of mIL-10 secreted by pTRmIL-10.

It was subsequently observed that *L. lactis* growth and lactic acid production remain unaffected at pH 8.5 providing that bacterial cultures are inoculated during logarithmic phase of growth. Buffering pTRmIL-10 cultures to 8.5 allowed for enhanced

production of mIL-10 *in vitro* providing mIL-10 was collected prior to its degradation (pH 6.5). Thus, pTRmIL-10 supernatants were collected 5 hours post-inoculation (pH ~7).

Survival of pTRmIL-10 and pTR1NX In Vivo

From erythromycin-resistant standard plate count results, it was clear that pTRmIL-10 and pTR1NX survived the harsh acidic conditions encountered in the stomach. Animals treated with pTR1NX and pTRmIL-10 exhibited 10^4-10^5 CFU/g of digesta in the stomach, a 10-100 fold increase over the 1000 bacterial cells which reportedly colonize the mammalian intestine at any one time (Fig 4 & 5; Gionchetti et al, 2000). As expected, these numbers increased as the bacteria entered the more amenable confines of the distal small intestine and colon.

Steidler and colleagues have shown that the gastric pH of mice treated with pTRmIL-10 and pTR1NX increases from 1.5 to 6 immediately following gavaging, dropping to pH 4 by 5 minutes and to 2.5 by 60 minutes. As gastric pH was not recorded over the course of this study, it is difficult to determine how these results correlate to the BBdp model. However, we can infer from the high numbers *L. lactis* in the stomach and proximal SI that a similar situation occurred in the BBdp rat. One possible explanation for this effective transfer is that the large volume of gavaging medium (pH ~7.5) in which the bacterium was delivered may neutralize stomach acid. Such an increase would provide a brief opportunity for gastric transit of *L. lactis* to the more amenable confines of the distal SI and colon. The acid-resistant nature of *Lactococcus lactis* would also complement this increase, allowing for the effective transfer of pTRmIL-10 and pTR1NX into the gut lumen.

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It is of note that pTR1NX was absent in the stomach of diabetic animals (Fig 4). One plausible explanation for this result is that animals with overt diabetes were fasted for 24 hours subsequent to their sacrifice. Thus, residual pTR1NX in the stomach would not have benefited from the buffering capacities of food. By contrast, pTRmIL-10 levels remained normal in diabetic animals, suggesting that pTRmIL-10 may have been more acid resistant than pTR1NX, affording it more protection as it encountered stomach acid. This claim is supported by the apparent 10-fold increase of pTRmIL-10 levels in the stomach of 45 day, 70 day and end-of-study animals compared to pTR1NX-treated animals (Fig 4 & 5).

Quantification of mIL-10 secreted by pTRmIL-10 In Vivo

Because of the prolonged nature of this study, one difficulty present from the outset was the accurate quantification of IL-10 production *in situ*. Steidler *et al* accomplished this task by treating control mice with 7 serial inocula containing 3.4×10^9 CFU of pTRmIL-10 every hour. Animals were sacrificed 30 minutes following the final inoculation and the intestine divided into anatomic compartments. Homogenized tissue from each compartment was plated to determine the CFU values of pTRmIL-10 and screened by ELISA to determine mIL-10 levels. Finally, back calculations were used to estimate total delivery of mIL-10.

There are many difficulties associated with extrapolating mIL-10 concentrations from such a procedure. Foremost, animals were gavaged with 2.3×10^{10} CFU of pTRmIL-10 over a 7 hour period, compared to 1×10^7 CFU of pTRmIL-10 delivered per day throughout the course of this study. This procedure does not account for increased survival of pTRmIL-10 associated with higher stomach pH and assumes that *in vitro* IL-

10 production would be independent of the quantity of pTRmIL-10 delivery or the time between gavages. Given these limitations, mIL-10 was not quantified in the intestinal lumen of BBdp rats. However, the quantity of the bacteria isolated from animals provides circumstantial evidence that mIL-10 production would have been substantial.

Increased Disease Incidence in BBdp Animals Treated With pTRmIL-10

Given the dichotomy between diabetes incidence observed in pTRmIL-10-treated animals (86.5%) and animals gavaged with pTRmIL-10 and p663 (68%), we investigated the possibility that one (or more) proteins expressed by pTRmIL-10 might be implicated in the high disease incidence (Fig 5). Given the lack of species specificity of mIL-10 delivered via pTRmIL-10 into the BBdp rat, it was reasoned that immunoreactivity against mIL-10 might be involved.

The subsequent observation that serum antibodies collected from animals treated with pTRmIL-10 were reactive against protein isolated from pTRmIL-10 supernatants but not against commercial mIL-10 suggests that an immune response may have been elicited against proteins constitutively expressed by the pTRmIL-10 vector or protein by-products of *L. lactis* but not against mIL-10 itself (Fig 7). A corresponding dot blot (data not shown) showed that individual and pooled serum collected from animals treated with pTRmIL-10 was not reactive against *L. lactis* lysate, suggesting that this response was a result of reactivity against proteins constitutively produced by pTRmIL-10.

To further explore this idea, serum collected from animals treated with pTRmIL-10 was pre-absorbed with 40-80 ng/mL of mIL-10 to bind any potential anti- mIL-10 antibodies present in the serum. Once again, the reactivity of this preabsorbed serum to pTRmIL-10 protein isolate but not against commercial mIL-10 further confirms that an

immune response was elicited against constitutive proteins secreted by pTRmIL-10 but not against mIL-10 itself (Fig 8).

mIL-10 and rat IL-10 Quantification In Vivo in BBdp Rats

Given the trend observed in animals treated with pTRmIL-10 towards increased T1D incidence and the absence of mIL-10- reactive antibodies, it was necessary to ascertain whether mIL-10 was able to reach its therapeutic target; namely auto-reactive T lymphocytes housed within the gut-associated lymphoid tissue (GALT). The GALT is comprised of a range of tissues including the lamina propria, Peyer's patches and intestinal epithelial cells (IEC) which house large numbers of lymphocytes. Steidler, et al have proposed that mIL-10 may reach these target tissues by one of two mechanisms. The first proposes that mIL-10 is secreted *in vivo* in the gut lumen and readily diffuses across the mucosal lining to the GALT. This passive transport would be facilitated by the increased inflammation and permeability observed in IBD and T1D models. A second, more novel proposal is that *Lactococcus* may be actively transported across the mucosal membrane where mIL-10 would be produced *in situ*.

The lack of mIL-10 in the serum of animals treated with pTRmIL-10 suggests that mIL-10 did not cross the mucosal barrier. Because mIL-10 was not quantified in the gut lumen, one cannot rule out the possibility that mIL-10 production was so low as to render it undetectable in the serum. Given that the bacterial solutions were not thawed until 30 minutes prior to gavaging, it is likely that pTRmIL-10 would have produced only minimal amounts of mIL-10 in the proximal small intestine but that these levels would have increased as the bacteria passed into the lower small intestine and colon.

Another possibility is that mIL-10 may be quickly degraded upon entry into the circulatory system. A subsequent study by Steidler *et al* provides merit to this explanation as it was observed that mIL-10 was undetectable in the serum of IL-$10^{-/-}$ mice treated with pTRmIL-10. A third explanation could be that mIL-10 may not exert its effect on cells within the GALT but rather on intestinal epithelial cells which are recognized as playing a key role in the gut immune response (Gewirtz, 2003). Given the apparent link between luminal antigens and T1D and IBD, it is conceivable that mIL-10 could alter disease incidence without leaving the confines of the gut lumen.

Impact of In Vivo Secretion of mIL-10 on Cytokine Expression

Results obtained from semi-quantitative RT-PCR analysis of duodenal tissue collected from BBdp rats treated with Buffer alone, p663, pTR1NX and pTRmIL-10 do not indicate a Th1 bias in the proximal small intestine of BBdp or BBd rats, contradicting the results of previous studies from our lab (Wang et al, 2003). IFN-γ expression, indicative of a Th1 bias, was low in all groups with the exception of diabetic animals treated with buffer alone and in end-of-study animals treated with p663. Although it is unclear what caused low IFN-γ expression, *L. lactis* and the gavaging buffer are two variables which were introduced in this study which may be implicated in this down-regulation. What is clear is that with the exception of animals treated with buffer alone, low expression of IFN-γ did not lead to a reduction in disease severity. This gives merit to the claim that the balance between Th1 and Th2 cytokines may be the overriding factor implicated in triggering disease onset, rather than individual cytokines.

Another interesting observation from the RT-PCR analysis of the duodenal tissue is that 70 day animals treated with Buffer alone exhibited increased IL-10 levels. This is

of note given that animals fed buffer alone had the lowest incidence of diabetes at 44%, suggesting a protective effect of the Buffer. An explanation for this apparent protection is unclear. However, it could be attributed to the casein component of the M17 bacterial medium (Appendix 1) or to glycerol in the Buffer. A similar increase in the expression of the Th2 cytokine IL-4 was also observed in 45 day old animals treated with p663. However, expression dropped significantly by 70 days of age and did not confer protection to these animals.

There are several possible explanations to the apparent inability of mIL-10 to alter cytokine expression in animals treated with pTRmIL-10. First, even though dot blot analysis suggests that animals did not develop an immune response against mIL-10, it is possible that lack of species specificity prevented mIL-10 from influencing cytokine expression in the gut. A second explanation is that pTRmIL-10 may not have produced mIL-10 in sufficient quantities in the proximal duodenum to alter *in vivo* expression of gut cytokines. Given that the gavaging medium was not thawed until immediately prior to treatment, it is likely that pTRmIL-10 would have directed its metabolic activities towards survival and replication rather than to the production of mIL-10, especially through the inhospitable environment of the stomach and proximal intestine. Furthermore, as pTRmIL-10 is delivered in an aqueous solution, it would have traveled rapidly through the proximal GI tract. Even if pTRmIL-10 was producing mIL-10 in the duodenum, amounts would be minimal and stomach acid and pancreatic enzymes would quickly destroy mIL-10. This explanation agrees with the results of Steidler and colleagues who found that mIL-10 was detectable only in the colon of IL-10 $^{-/-}$ mice even though pTRmIL-10 was present throughout the gut.

A Role for the Intestinal Flora in T1D?

Perhaps the most novel finding of this study is the increase in *L. lactis* levels observed in BBdp animals immediately following onset of T1D. Further data, derived from a previous study conducted in our lab suggest that levels of *Lactobacillus*, *Enterococcus*, *Staphylococcus* and Coliforms are also increased in overt diabetic animals (Fig 12). Although these results do not ascertain when this increase occurs, they provide preliminary data suggesting a link between alterations in the gut bacteria and T1D. Furthermore, it is interesting to note that with the exception of *Lactobacillus* and *Lactococcus*, all of these bacterial species are opportunistic pathogens. This implies that a microbial imbalance leading to opportunistic infection could be associated with development of T1D. To further examine this possibility, it is prudent to look at the striking similarities that exist between these results and those observed in the IBD model.

IBD is associated with a hyperactive immune response against commensal bacterial antigens in the gut. Spontaneous colitis, which develops in IL-2-, IL-10- and TCR-knockout mice fails to develop when animals are raised in germ-free conditions. Furthermore, treatment with antibiotics such as metronidazole, ciprofloxacin, or neomycin have been shown to prevent colitis in IL-$10^{-/-}$ mice prior to disease onset and attenuate disease severity in the same model as well as in human IBD patients (Madsen et al, 2000; Sutherland et al 1991; Turunen et al, 1998).

Characterization of the gut flora of IBD models further implicates overgrowth of the autochthonous microflora in triggering disease onset, in particular, a rise in the number of opportunistic pathogens. It has long been known that patients with Crohn's disease (CD) and ulcerative colitis (UC) exhibit increased concentrations of antibodies

against a variety of indigenous bacterial species including *Bacteroides fragilis*, *Streptococcus faecalis* and numerous *Peptostreptococcus*, *Eubacterium*, and *E. coil* species and subtypes. Studies have also shown that levels of *E. coli, Bacteroides fragilis, Bacteroides vulgatus,* and *Mycobacterium paratuberculosis* are increased prior to clinical diagnosis of IBD (Elson, et al 1995; Schultsz et al, 1997; Darfeuille-Michaud et al, 1998; Engstrand, 1995; Giaffer et al, 1991). Finally, it has recently been shown that patients suffering from IBD and mice with experimental UC have $CD4^+$ T cells reactive against bacteria such as *Enterobacteria, Bacteroides* and other bacterial antigens present in the intestinal lumen (Duchmann et al, 1999; Cong et al, 1998).

This apparent overgrowth of pathogens appears to coincide with a decrease in non-pathogenic, gram positive Lactic Acid Bacteria (LAB) which play a pivitol role in maintaining a healthy, balanced normal flora. Characterization of the fecal extracts of patients suffering from Crohn's disease show a decrease in *Bifidobacterium* and β-galactosidase levels, leading to impaired breakdown of complex carbohydrates. It has also been observed that *Lactobacillus spp* are decreased in the colon of IL-$10^{-/-}$ mice suffering from UC (Madsen et al, 1999).

The importance of a balanced normal flora in prevention of IBD is underscored by the fact that oral treatment with the probiotics *Lactobacillus reuteri* and *L. plantarum* has been shown to prevent UC in IL-$10^{-/-}$ mice while treatment with *Bifidobacterium* and *Streptococcus thermophilus* has been shown to beneficially affect patients suffering from IBD (Pathmakanthan et al, 1999; Schultz et al, 2002; Fabia et al, 1993; Favier et al, 1997; Giaffer et al, 1991; Madsen et al, 1999; Schultz et al, 1998).

Although data linking microbial imbalance and T1D is very limited, one study is of note. Matsuzaki and collegues have observed that oral treatment of the heat-killed probiotic *Lactobacillus casei* decreased diabetes incidence in the NOD mouse model by ~ 60%. Treatment corresponded with a shift towards a Th2 response characterized by increased production of IL-4 and IL-10 and a decrease in IFN-γ levels (Matsuzaki et al, 1997). When combined with the results of our present study, these results suggest that modification of the gut flora may be important in prevention of T1D in both the NOD and BBdp animal model. Further study is necessary to evaluate this possibility.

Given the results which implicate dietary modification and disease incidence, it is possible that diet could play a role in modification of the gut flora. Historically, diabetes incidence in the BBdp rat is significantly higher in animals fed with an unpurified wheat-based diets such as NIH-07 (63.0 ± 13%; n=8 experiments, total of 145 rats) or NTP-2000 (65.3 ± 14.9 %; n=6 experiments, total of 169 rats) compared to animals fed a semi-purified hydrolyzed casein diet (18.8 ± 10.6%; n=14 experiments, total of 322 rats; Scott 1996; MacFarlane, et al, 2003). Furthermore, it is well documented that bacterial growth within the gut lumen can be altered through dietary modification. Therefore, certain diets may provide protection against the disease by influencing control over the endogenous flora.

It is unclear how dietary modification and bacterial overgrowth may lead to the initiation of an increased immune response to dietary antigen. Current thought is that wheat proteins may be actively transported across the epithelial barrier and presented to $CD4^+$ T helper cells in the lamina propria. One must also consider the impact that permeability defects may have on antigen presentation. The healthy gut maintains a strict

yet fragile barrier between the host's immune cells and the vast bacterial ecosystem and dietary antigens present in the gut. Control over this system is maintained via complex interactions between the autochthonous flora, specific and non-specific physiological defence mechanisms such as mucous secretion and peristalsis, and the innate immune system. However, regulation of the intestinal epithelium minimizes direct interactions between luminal antigen and the host's immune effector cells. Thus, it is possible that increased gut permeability observed in T1D patients may compromise this barrier, and allow increased influx of dietary and bacterial antigens, including whole bacteria (Meddings et al, 1999; Hardin et al, 2002). This would provide an opportunity for direct contact between antigen and immune effector cells, triggering reactivity against these antigens. These effector cells could then traffic to the pancreas where some stimuli triggers attack of β-cells, leading to a cascade of events culminating in T1D.

Summary

In conclusion, oral delivery of pTRmIL-10 appeared to increase T1D incidence in the BBdp rat compared with animals treated with Buffer alone but not in comparison with animals gavaged with control bacterium. Evidence suggests that immunoreactivity against proteins constitutively produced by pTRmIL-10 may have triggered this increase. Given the difficulty associated with mIL-10 quantification *in vivo*, it is impossible to determine the concentration of mIL-10 in the gut of live animals. The presence of pTRmIL-10 throughout the gut lumen provides circumstantial evidence that mIL-10 production would have been substantial, particularly in the distal small intestine and colon. However, mIL-10 and rat IL-10 was not detectable in serum, suggesting that mIL-10 did not cross the mucosal barrier or was degraded in the gut lumen prior to transport.

No conclusive statement can be made regarding the impact of pTRmIL-10 on gut Th1/Th2 cytokine balance as a Th1-skewed cytokine profile was not observed in the duodenal tissue of these animals. However, it is of note that 70 day old animals treated with Buffer alone exhibited increased IL-4 expression, indicative of a Th2 cytokine bias. Furthermore, these animals had a disease incidence of 44%, suggesting that oral delivery of gavaging medium alone provided protection from the disease.

Finally, it was observed that diabetic animals exhibited increased concentrations of various bacterial species of the gut microflora, including *L. lactis* pTRmIL-10 and pTR1NX. This suggests that bacterial overgrowth may be associated with early stages of diabetes. Further studies will be necessary to elucidate the impact of bacterial overgrowth to determine if it triggers disease onset (cause) or is a complication arising immediately following overt diabetes (effect).

Although oral delivery of pTRmIL-10 did not attenuate T1D incidence as hypothesized, the results from this study further suggest that interactions between the mucosal immune system and dietary and possibly bacterial antigens within the gut lumen are associated with T1D and may be responsible for triggering disease onset.

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Appendix

Appendix 1

M17 Formulation (per litre): Pancreatic digest of casein, 5g; Soy peptone, 5g; Beef extract, 5g; Yeast extract, 2.5g; Ascorbic acid, 0.5g; Magnesium sulfate, 0.25g; Disodium-β-glycerophosphate, 19g.

Appendix 2

M9 Minimal Salts Formulation, 5X (per litre): Sodium phosphate, dibasic, anhydrous, 33.9g; Sodium phosphate, monobasic, 15g; Sodium chloride, 2.5g; Ammonium chloride, 5g.

Appendix 3

Sample Calculation of Counting MC/9 Cells using the Trypan Blue Exclusion Method: 124 cell average (count both sides of hemocytometer; average) × 10 (dilution factor) × 10^4 (multiplication factor of hemocytometer to determine the number of cells/mL) = 1.24 × 10^7 cells/mL

Appendix 4

Sample Calculation of Standard Plate Count technique: 45 colonies/plate ÷ 0.1 mL/plate (volume of bacterial suspension added per plate) × 10^5 (dilution factor) × 0.76 g digesta = 3.42 × 10^7 CFU/g digesta

Appendix 5

Primer sequence:

IL-10: sense: 5'-TGCCTTCAGTCAAGTGAAGACT-3', anti-sense: 5'-AAACTCATTCATGGCCTTGTA-3';

IFN-γ: sense: 5'-CGTCTTGGTTTTGCAGCTC-3', anti-sense: 5'-ACTCCTTTTCCTCTTCCTTA-3';

IL-4: sense: 5'-CTTGCTGTCACCCTGTTC-3', anti-sense: 5'-CATGGAAGTGCAGGACTGCA-3';

β-actin: sense: 5'-CCAGCCTTCCTTCCTGGGTA-3', anti-sense: 5'-CTAGAAGCATTTGCGGTGCA-3'.

Appendix 6

Image analysis of IL-10: Image analysis was used to semi-quantify total protein secreted by pTRmIL-10 as well as mIL-10 itself. Net intensity of total protein = 647,381.22. Net intensity of mIL-10 protein = 169,845.31. (164,845.31/647,381.22) × 100 = 25.46% of pTRmIL-10 protein isolate is mIL-10

Figures

Figure 1. SDS PAGE analysis of *L. lactis* pTR1NX and pTRmIL-10 protein. Total protein (2.5 and 5 μg/well) was separated on a 15% SDS gel by electrophoresis (150 V, 109 mAmp) and developed using silver nitrate staining procedures. The arrow denotes mIL-10 (18.45 kDa). The molecular marker was purchased from Invitrogen.

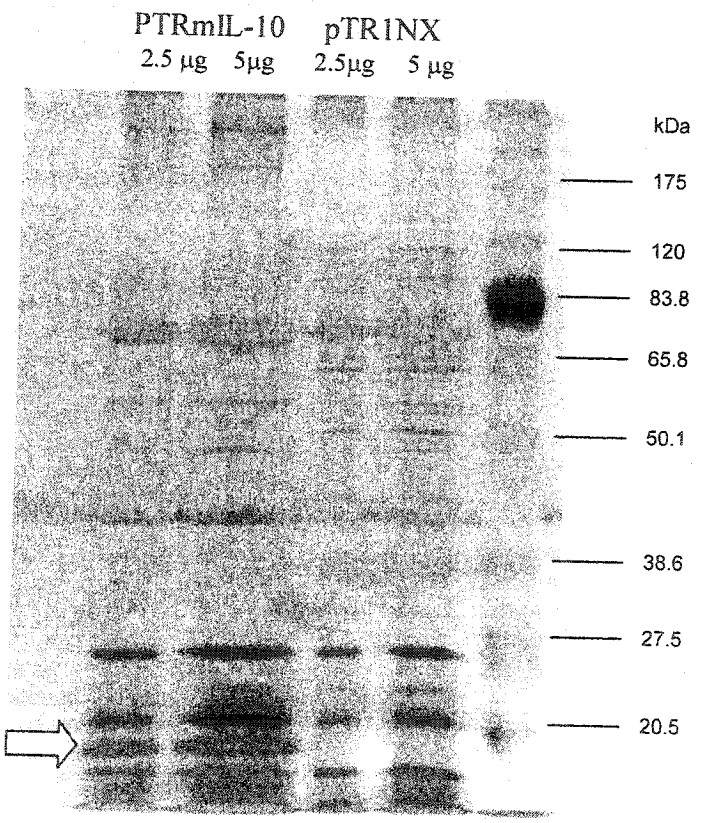

PTRmIL-10
2.5 µg
5µg
pTR1NX
2.5µg
5 µg
kDa
175
120
83.8
65.8
50.1
38.6
27.5
20.5

Figure 2. Western blot analysis of *L. lactis* pTR1NX and pTRmIL-10 protein isolate. mIL-10, pTR1NX and pTRmIL-10 protein isolates were separated on a 15% SDS gel by electrophoresis (150 V, 109 mAmp). Samples were probed with rat anti-mIL-10 antibody (1:1500) and labeled with alkaline-phosphatase-conjugated goat anti-rat IgG antibody (1:1500). The arrow denotes mIL-10 (18.45 kDa). The molecular marker was purchased from Invitrogen.

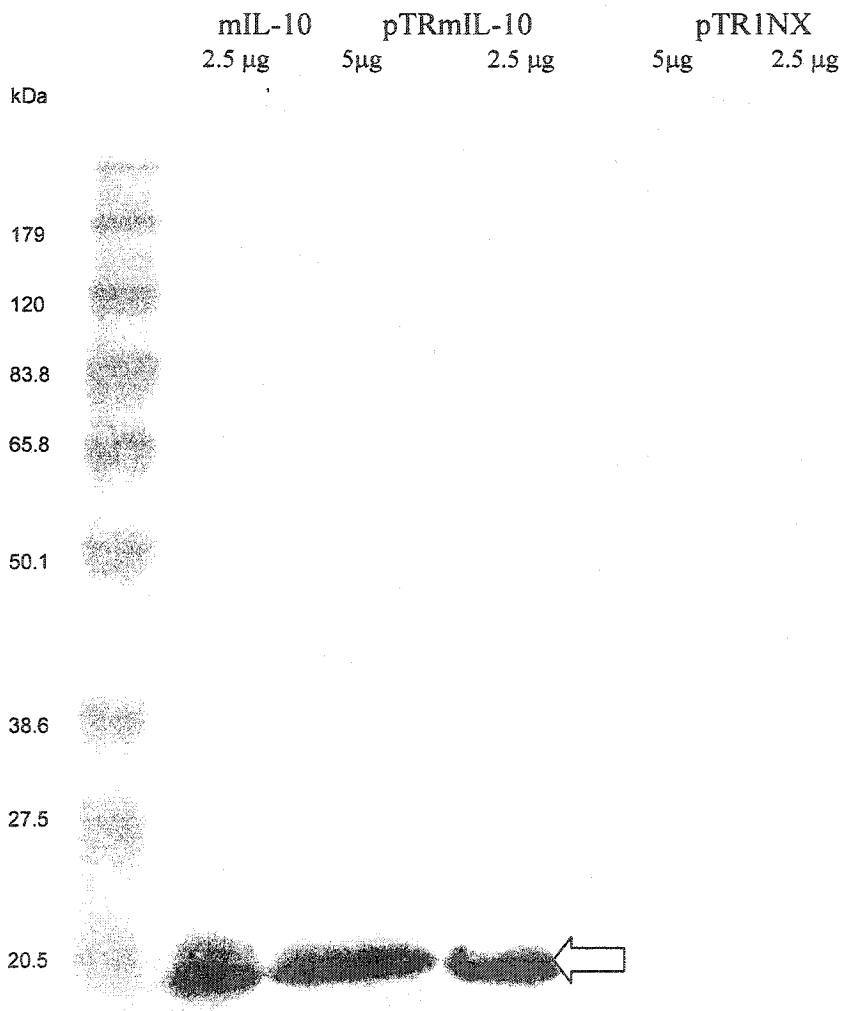

mIL-10
2.5 µg
pTRmIL-10
5µg
2.5 µg
pTR1NX
5µg
2.5 µg
kDa
179
120
83.8
65.8
50.1
38.6
27.5
20.5

Figure 3. MC/9 assay of biological activity of *L. lactis* pTRmIL-10 isolate. MC/9 cells were co-incubated with mIL-4, mIL-10 or pTRmIL-10 isolate for 72 hours prior to counting using the Trypan Blue exclusion method (n = 8). Bars (mean +/- SE) sharing the same letter are statistically different ($p \leq 0.05$) as determined by ANOVA (Fisher LSD post-hoc analysis).

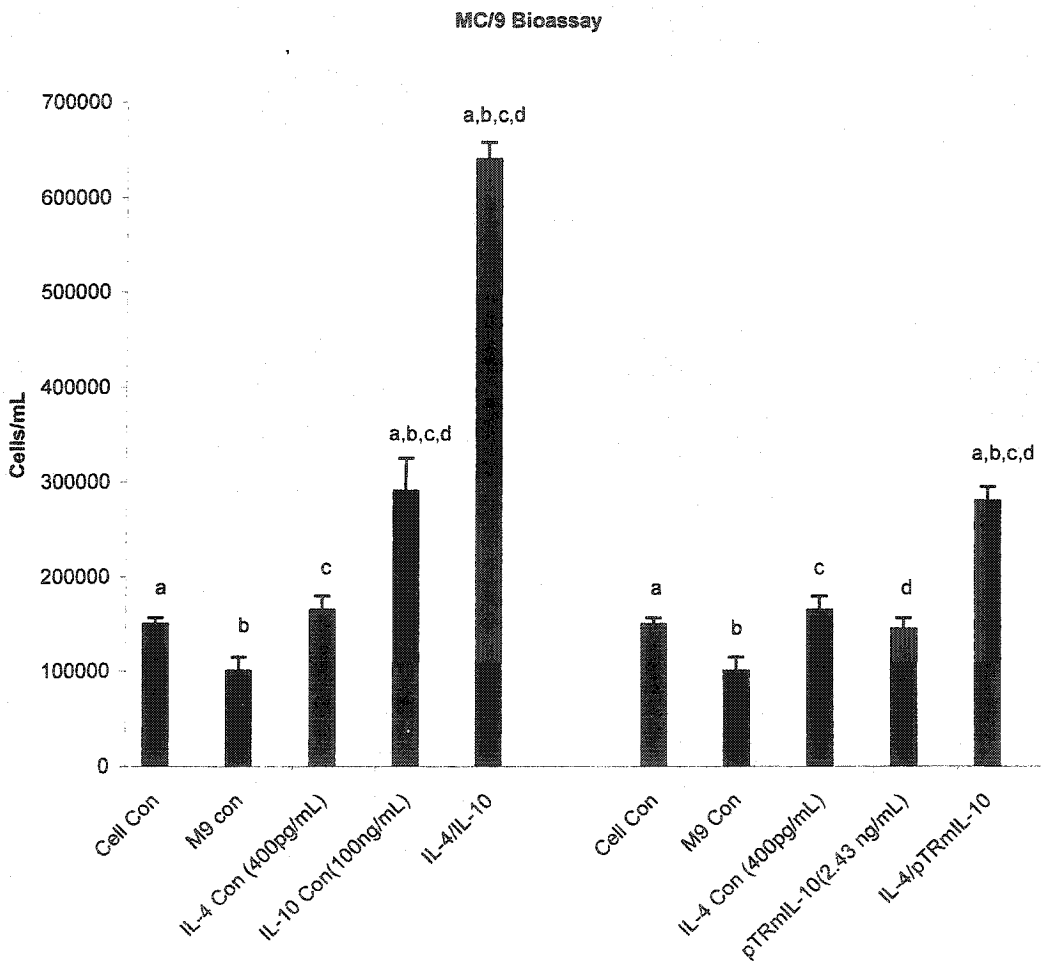

MC/9 Bioassay
Cells/mL
700000
600000
500000
400000
300000
200000
100000
0
a
b
c
a,b,c,d
a,b,c,d
a
b
c
d
a,b,c,d
Cell Con
M9 con
IL-4 Con (400pg/mL)
IL-10 Con(100ng/mL)
IL-4/IL-10
Cell Con
M9 Con
IL-4 Con (400pg/mL)
pTRmIL-10(2.43 ng/mL)
IL-4/pTRmIL-10

Figure 4. Erythromycin-selective plate counts of the gastrointestinal content of BBdp animals fed *L. lactis* pTR1NX. Digesta/fecal material were serially diluted and plated on M17 plates containing 5 μg/mL of erythromycin. Plates were incubated at 30 °C for 24-48 hours before counting. Bars (mean +/- SE) sharing the same letter are statistically different ($p \leq 0.05$) as determined by ANOVA (Fisher LSD post-hoc analysis).

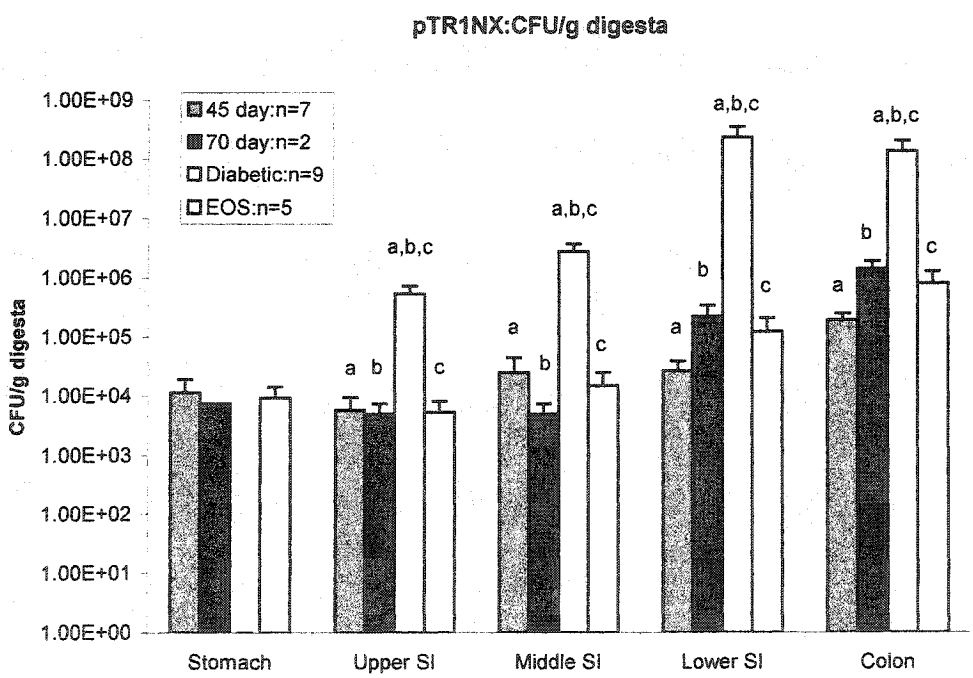

pTR1NX:CFU/g digesta
45 day:n=7
70 day:n=2
Diabetic:n=9
EOS:n=5
CFU/g digesta
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
a,b,c
a,b,c
a,b,c
a b
a b
a b
c
c
c
a
b
c
Stomach
Upper SI
Middle SI
Lower SI
Colon

Figure 5. Erythromycin-selective plate counts of the gastrointestinal content of BBdp animals fed *L lactis* pTRmIL-10. Digesta/fecal material were serially diluted and plated on M17 plates containing 5 μg/mL of erythromycin. Plates were incubated at 30 °C for 24-48 hours before counting. Bars (mean +/- SE) sharing the same letter are statistically different ($p \leq 0.05$) as determined by ANOVA (Fisher LSD post-hoc analysis).

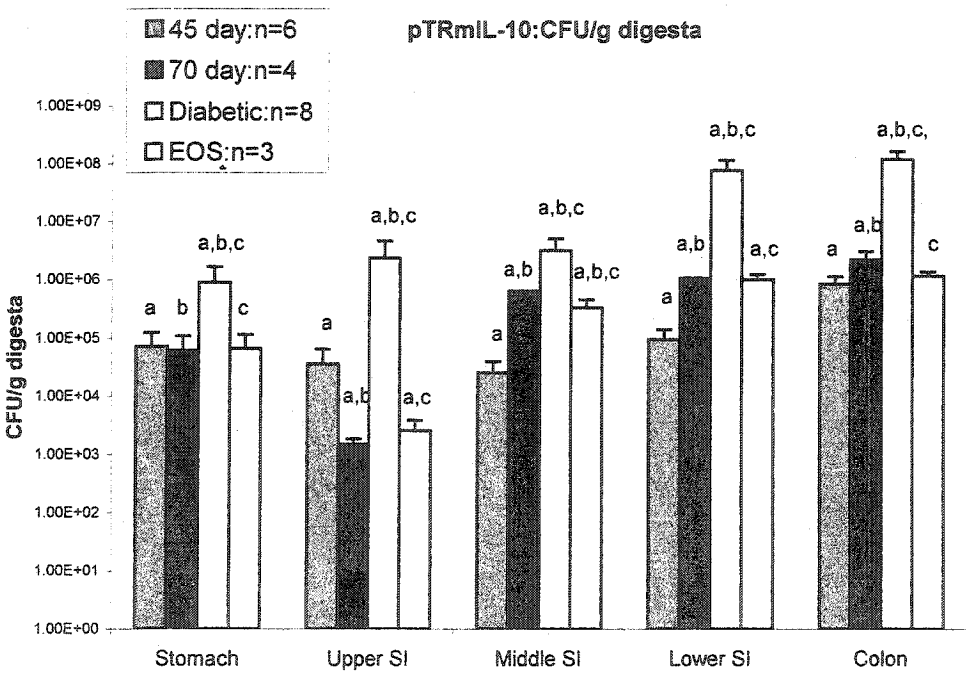

45 day:n=6
70 day:n=4
Diabetic:n=8
EOS:n=3
pTRmIL-10:CFU/g digesta
CFU/g digesta
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
a
b
a,b,c
c
a
a,b
a,b,c
a,c
a
a,b
a,b,c
a,b,c
a
a,b
a,b,c
a,c
a
a,b
a,b,c,
c
Stomach
Upper SI
Middle SI
Lower SI
Colon

Figure 6. Survival graph of BBdp rats gavaged with *L. lactis* pTRmIL-10. Animals were fed a diabetes-promoting NTP-2000 diet and were gavaged daily from 26 days until diagnosis of overt diabetes, or 160 days. n= 25 except pTRmIL-10 where n = 22. For comparisons of final diabetes incidence, buffer vs pTRmIL-10 = p 0.0054; Buffer vs p663 or pTR1NX = p 0.4783 and pTRmIL-10 vs p663 or pTR1NX = p 0.6606 as determined by Fisher Exact test.

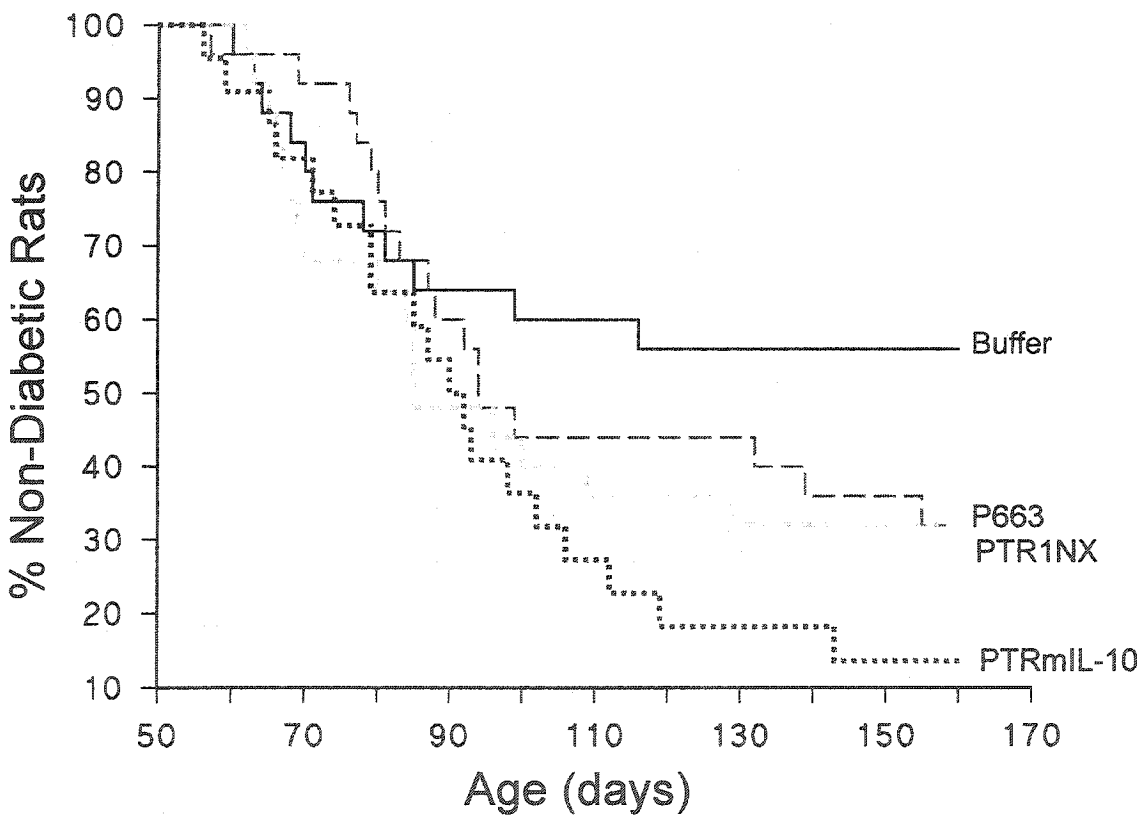
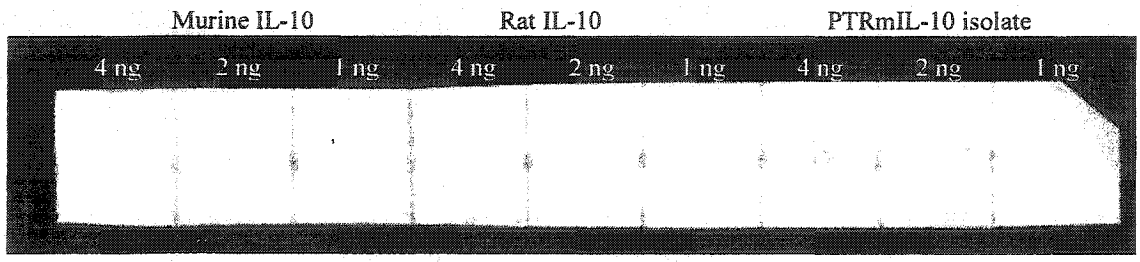

% Non-Diabetic Rats
100
90
80
70
60
50
40
30
20
10
50
70
90
110
130
150
170
Age (days)
Buffer
P663
PTR1NX
PTRmIL-10

Figure 7. Positive control dot-blot of anti-mIL-10 used to probe mIL-10, rat IL-10 and pTRmIL-10 isolate: Rat anti-mIL-10 (1:2000) was used to probe a serial dilution of commercial murine and rat IL-10 and pTRmIL-10 protein isolate. The secondary antibody was goat anti-rat IgG conjugated with alkaline phosphatase (1:1500).

Figure 8. Pre-absorbed dot-blot using pooled sera collected from animals treated with pTRmIL-10 against mIL-10, rat IL-10 and pTRmIL-10 isolate: Pooled sera from animals treated with pTRmIL-10 (1:50) was used to probe murine and rat IL-10 and pTRmIL-10 protein isolate. Serum was pre-absorbed with 40 and 80 ng/mL of mIL-10 as shown in lane 2 and 3. Lane 1 is unabsorbed serum. The secondary antibody is goat anti- rat IgG conjugated with alkaline phosphatase (1:1500).


Murine IL-10
Rat IL-10
PTRmIL-10 isolate
4 ng
2 ng
1 ng
4 ng
2 ng
1 ng
4 ng
2 ng
1 ng

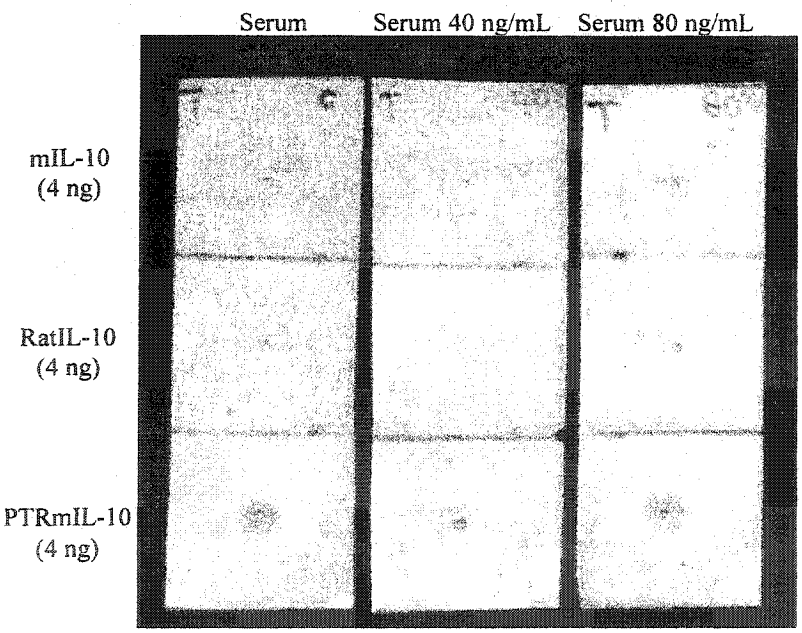
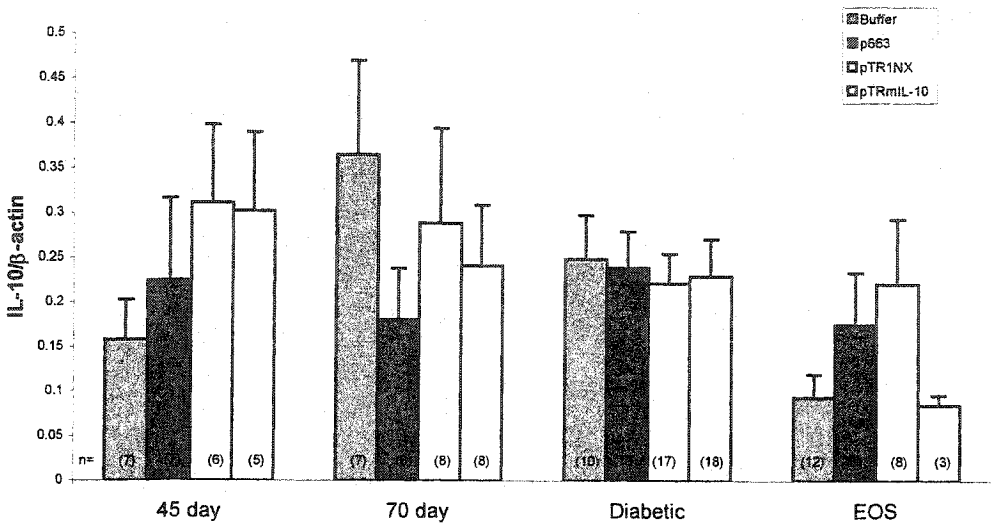

Serum
Serum 40 ng/mL
Serum 80 ng/mL
mIL-10
(4 ng)
RatIL-10
(4 ng)
PTRmIL-10
(4 ng)

Figure 9. Semi-quantitative RT-PCR analysis of IL-10 mRNA in duodenum of rats treated with Buffer, p663, pTR1NX and pTRmIL-10. Bars (mean +/- SE) sharing the same letter are statistically different ($p \leq 0.05$; Fisher LSD post-hoc analysis). Numbers in brackets denote the number of animals per experiment.

A


IL-10/β-actin: Duodenal RNA
IL-10/β-actin
Buffer
p663
pTR1NX
pTRmIL-10
n=
45 day
70 day
Diabetic
EOS

B

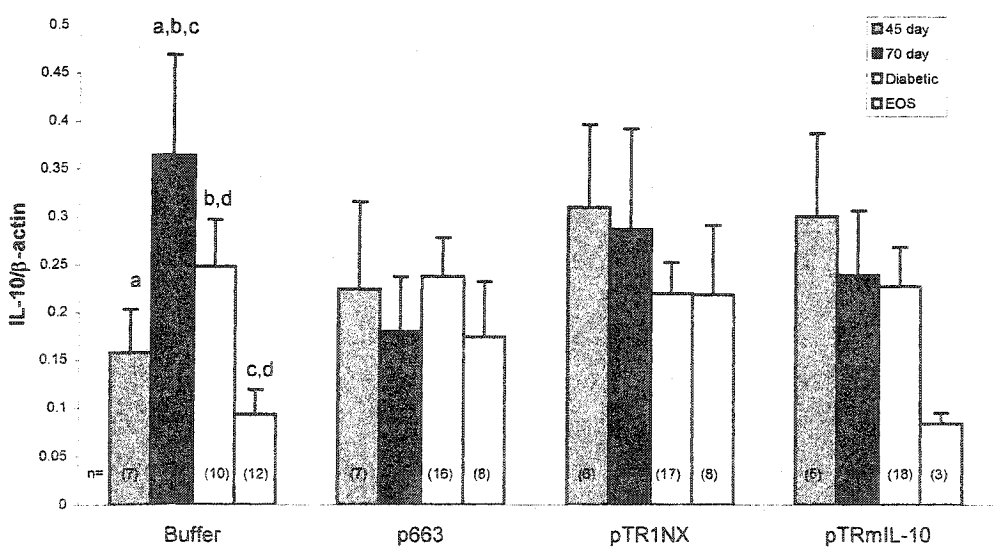
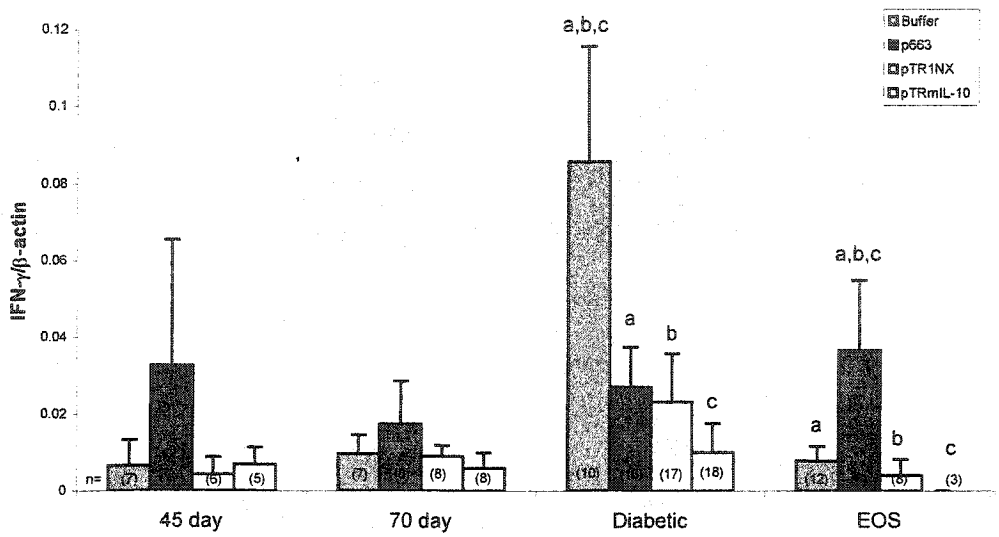

IL-10/β-actin
a,b,c
a
b,d
c,d
45 day
70 day
Diabetic
EOS
n=
Buffer
p663
pTR1NX
pTRmIL-10

Figure 10. Semi-quantitative RT-PCR analysis of INF-γ mRNA in duodenum of rats treated with Buffer, p663, pTR1NX and pTRmIL-10. Bars (mean +/- SE) sharing the same letter are statistically different ($p \leq 0.05$; Fisher LSD post-hoc analysis). Numbers in brackets denote the number of animals per experiment.

A


IFN-γ/β-actin: Duodenal RNA
IFN-γ/β-actin
0.12
0.1
0.08
0.06
0.04
0.02
0
Buffer
p663
pTR1NX
pTRmIL-10
a,b,c
a
b
c
a,b,c
a
b
c
n=
(7)
(5)
(8)
(8)
(10)
(17)
(18)
(12)
(6)
(3)
45 day
70 day
Diabetic
EOS

B

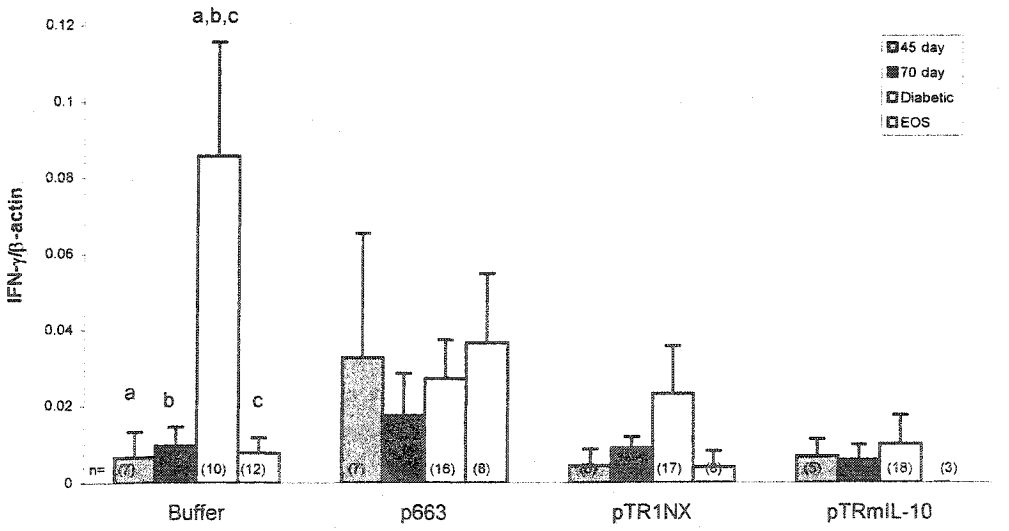
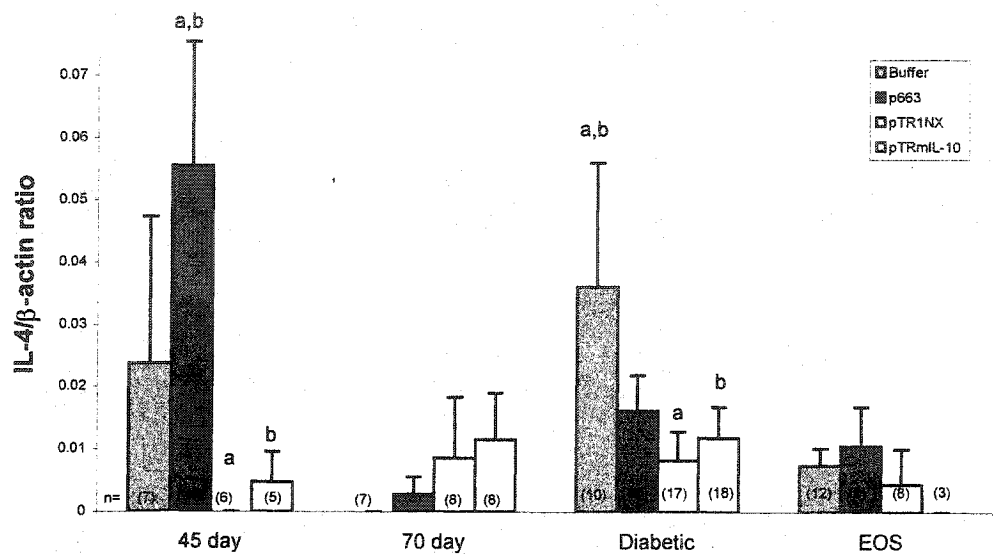

IFN-γ/β-actin
0.12
0.1
0.08
0.06
0.04
0.02
0
45 day
70 day
Diabetic
EOS
a,b,c
a
b
c
n=
(7)
(10)
(12)
(7)
(16)
(8)
(17)
(6)
(5)
(18)
(3)
Buffer
p663
pTR1NX
pTRmIL-10

Figure 11. Semi-quantitative RT-PCR analysis of IL-4 mRNA in duodenum of rats treated with Buffer, p663, pTR1NX and pTRmIL-10. Bars (mean +/- SE) sharing the same letter are statistically different ($p \leq 0.05$; Fisher LSD post-hoc analysis). Numbers in brackets denote the number of animals per experiment.

A


IL-4/β-actin: Duodenal RNA
IL-4/β-actin ratio
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Buffer
p663
pTR1NX
pTRmIL-10
a,b
a
b
a,b
a
b
n=
(7)
(6)
(5)
(7)
(8)
(8)
(10)
(17)
(18)
(12)
(8)
(3)
45 day
70 day
Diabetic
EOS

B

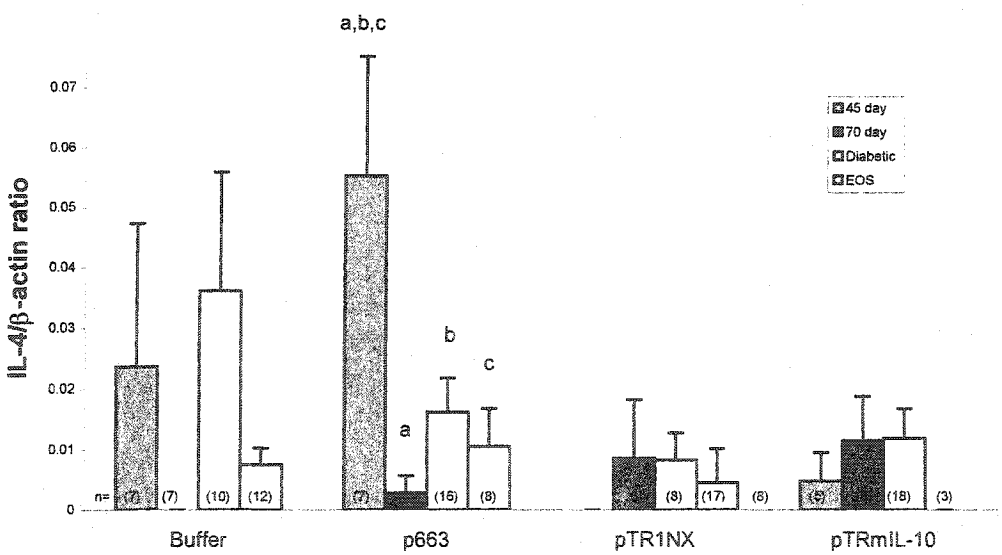
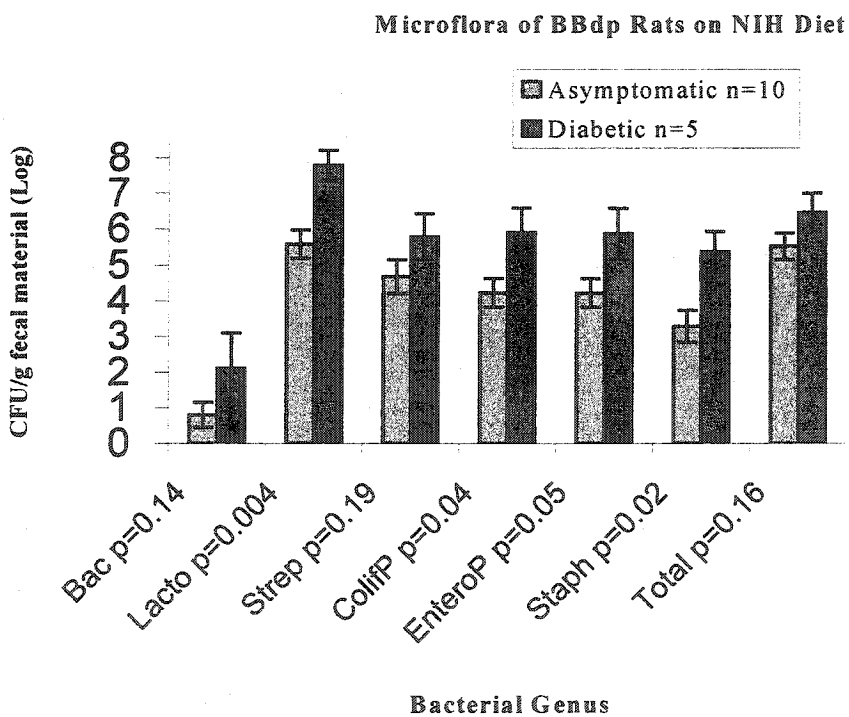

IL-4/β-actin ratio
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
45 day
70 day
Diabetic
EOS
a,b,c
a
b
c
n=
(7)
(7)
(10)
(12)
(7)
(15)
(8)
(8)
(17)
(6)
(18)
(3)
Buffer
p663
pTR1NX
pTRmIL-10

Figure 12. Comparisons of the intestinal microflora of asymptomatic and diabetic BBdp rats fed a diabetes-promoting NIH-07 diet. BBdp rats were fed an NIH-07 diet and were sacrificed upon diagnosis of the disease or at 150 days of age. Standard plate counts (mean +/- SE) were performed on fecal extracts to quantify various bacterial species. Statistical analysis was performed by ANOVA using Fisher LSD post-hoc analysis ($p \leq 0.05$).


Microflora of BBdp Rats on NIH Diet
Asymptomatic n=10
Diabetic n=5
CFU/g fecal material (Log)
0
1
2
3
4
5
6
7
8
Bac p=0.14
Lacto p=0.004
Strep p=0.19
ColifP p=0.04
EnteroP p=0.05
Staph p=0.02
Total p=0.16
Bacterial Genus

Scientific Skills

Advanced techniques learned in mammalian cell culture systems (Level 2), bacterial culturing and identification, bacterial binding assays, one-dimensional SDS-PAGE, Western blot/Dot blot analysis, protein/nucleic acid/plasmid isolation, semi-quantitative RT-PCR analysis, Bioassay/ELISA optimization, scanning electron microscopy, light microscopy, serum antibody detection and image analysis. Experience using statistical programs SPS and Statistica and graphing program SigmaPlot.

Presentations and Publications

- Canadian Society of Microbiologists: Montreal, June 1999.
  Poster Presentation: *Scanning Electron Microscopy Study of Microbial Colonization and Biofilm Development of Toothbrush Bristle Surfaces.* Bailey, JM, H Taylor, R Roy, and T Wallace.
- American Association of Immunology: Seattle, May 2000.
  Poster Presentation: *Induction of Interleukin-6 and Tumour Necrosis Factor Production by Lactic Acid Bacteria.* Wallace TD, Measham JD, Tompkins TA and Green-Johnson JM
- Danone Symposium on Fermented Food, Fermentation and Intestinal Flora: New York City, May 2000.
  Poster Presentation: *Effects of Lactic Acid Bacteria on cytokine production by a human intestinal epithelial cell line.* Wallace TD, Tompkins TA and Green-Johnson JM.
- International Congress of Mucosal Immunology: Orlando, June 2002.
  Poster Presentation: *The effects of Fructo-oligosaccharides on the intestinal microflora and autoimmune diabetes in the diabetes prone BB rat.* Wallace TD, Austin J, Farber J and Scott FW.
- Journal of Food Protection: In Press
  Interactions of Lactic Acid Bacteria with Human Intestinal Epithelial cells: effects on Cytokine Production. Wallace TD, Bradley S, Buckley ND and Green-Johnson JM.

Volunteer Experience

Sensory Motor Intelligence Learning Experience (SMILE): 1997-1999.
This mentoring program paired Acadia University students one-on-one with physically, psychologically, and mentally challenged school age children.

Canadian Ski Patrol System: Gatineau Zone (CSPS): 2002-
This program provides on-mountain First-Aid/CPR services for ski resorts in the Gatineau/Eastern Ontario region. Volunteers are trained in the delivery of First-Aid/CPR (Level C), on-mountain evacuations and lift-evacuations.

Statement of Contribution of Collaborators

Dr. Lothar Steidler

Dr. Lothar Steidler provided *L. lactis* p663, pTR1NX and pTRmIL-10 for use in this study.