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Epithelial Cells: an Immune Modulator in the Context of Inflammatory Bowel Diseases

by

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To

My parents and husband for their unending love and support

Abstract

Inflammatory Bowel Diseases (IBD) result from the nexus of a genetic predisposition, dysregulated immunologic insult against commensal microflora, and an environmental trigger. The intestinal epithelium is a single cell layer that separates a highly active mucosal immune system from a large antigenic load in the intestinal lumen. Innate immune recognition combined with a highly regulated adaptive immune response maintains this tolerance. The intestinal epithelium in collusion with antigen presenting cells primarily modulates this activity. In this thesis, we show that, in response to DNA isolated from bacteria, innate toll like receptor 9 (TLR9) activation in intestinal epithelial cells modulates both arms of the immune system, to regulate intestinal homeostasis, and through this mechanism, *Bifidobacteria breve* DNA exerts its anti-inflammatory function.

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List of Abbreviations

AP-1	**Activator protein 1**
APCs	**Antigen presenting cells**
Card15	**Caspase recruitment domain 15**
CD	**Cluster of differentiation**
Crohn's	**Crohn's disease**
cDNA	**Complementary deoxyribonucleic acid**
CFSE	**Carboxyfluorescein succinimidyl ester**
CMF-PBS	**Calcium magnesium free- phosphate buffered saline**
DC	**Dendritic cell**
DMEM	**Dulbecco's modified Eagle's medium**
DNA	**Deoxyribonucleic acid**
DNP	**2,4-Dinitrophenol**
DSS	**Dextran sulfate sodium**
FACS	**Fluorescent-activated cell sorting**
FOXp3	**Forkhead box P3**
GI	**Gastrointestinal**
GM-CSF	**Granulocyte-macrophage colony-stimulating factor**
GWAS	**Genome wide association study**

HEPES	**4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid**
IBD	**Inflammatory bowel disease**
IEC	**Intestinal epithelial cells**
IFN	**Interferon**
IkB	**Inhibitor of kappa B**
IL	**Interleukin**
Jak/Stat	**Janus Kinase/ Signal Transducers and Activators of Transcription**
LPS	**Lippopolysaccharide**
MAPK	**Mitogen-activated protein (MAP) kinases**
MDP	**Muramyl dipeptide**
MCP-1	**monocyte chemotactic protein-1**
MHC	**Major histocompatability complex**
MLN	**Mesenteric lymph node**
MyD88	**Myeloid differentiation primary response gene (88)**
NFkB	**Nuclear factor kappa B**
NK	**Natural killer**
NOD	**Nucleotide-binding oligomerization domain**
PAMPs	**Pathogen-associated molecular patterns**
PBS	**Phosphate buffered saline**
PRRs	**Pattern recognition receptors**

Rag	**Recombinase activating gene**
RNA	**Ribonucleic acid**
SCID	**Severe combined immunodeficiency**
SDS	**Sodium Dodecyl Sulfate**
STAT	**Signal transducers and activators of transcription**
TCR	**T cell receptor**
Th	**T helper type**
TLDA	**Taqman low density array**
TLRs	**Toll like receptors**
TNF	**Tumor necrosis factor**
TSLP	**Thymic stromal lymphopoietin**
UC	**Ulcerative colitis**

Chapter 1. Introduction

1.1 Relevance and Scope

Inflammatory bowel disease (IBD) encompasses two diseases with unknown etiology; Crohn's disease (Crohn's) and ulcerative colitis (UC). UC is a continuous inflammatory disease of the colon with inflammation isolated to the mucosa. It is associated with a T-helper type 2 (Th2)-mediated immune response with cytokines Interleukin (IL) IL-13 and IL-4, mediated by specialized Natural killer (NK) T cells.[1] Crohn's is a patchy transmural disease that can occur anywhere in the gastrointestinal (GI) tract, with increased prevalence in the ileum. It is associated with a T-helper type 1(Th1), T-helper type 17(Th17)-mediated immune response with cytokines IL-6, IL-12/23, Interferon gamma (IFNγ), Tumor necrosis factor alpha (TNFα), and the classical inflammation cascade. The inflammation is associated with the mucosa, the underlying lamina propria, and smooth muscle.[2] These diseases present often within the second to third decade of life, though an increased prevalence is seen among younger generations, including pediatric patients.[3] Canada and the US have some of the highest rates of IBD with the prevalence increasing in some populations,[2] though it seems to have stabilized in Europe, the UK, and North America.[4] Due to the large numbers of affected individuals, the impact that these diseases have on quality of life and the strain that a lifelong disease has on the healthcare system, research is being focused on disease etiology and new treatments.

Inflammatory bowel diseases have three pathological components: environmental, genetic, and immunomodulatory, which must all be present for an individual to present signs of disease. Current research is focused on the genetic component and the immune dysregulation that occurs. Currently, at least 30 genes are associated with the diseases,[5] and genetic abnormalities in innate immune receptors (such as NOD2/Card15[6] and Toll-like receptors (TLRs)[6-8] have been found to be associated with IBD. TLR9 polymorphisms were found to be associated with Crohn's in a world-wide meta-analysis.[9] Similarly, *in vivo* and *in vitro* studies reveal that TLR9 signaling is needed for intestinal homeostasis.[8] TLR9 signaling was also shown to be necessary for the anti-inflammatory effects of the probiotic VSL#3, in a DSS-induced colitis mouse model.[10]

Intestinal epithelial cells (IEC) are the primary barrier between the host and the luminal microflora, bacterial products, and food antigens. Furthermore, IECs initiate and modulate immune responses to the luminal contents and maintain homeostasis in a normal host. Microbial host cross-talk is important in controlling inflammation and maintaining homeostasis.[11] IECs mediate host microbial interactions in production and secretion of antimicrobial peptides, defensins,[12] and in the production of chemokines and cytokines that recruit and activate innate and adaptive immune cells. Previously, our laboratory has shown that IECs are able to mount a differential response to probiotic, commensal, and pathogenic bacteria DNA in a TLR9-dependant way.[13] NFκB is a transcription factor associated with many immunological activities, including the production of

pro-inflammatory cytokines, which aid in the response to intestinal inflammation. Probiotic bacterial DNA has been shown to regulate the IEC immune response as it down-regulates the NFκB pathway by inhibiting the proteasome, resulting in an increase in NFκB inhibitor IκB.[14-15] The emerging role of IECs as immune modulators needs to be investigated in IBD.

Dendritic cells are the sentinels of the immune system, bridging innate and adaptive immune responses, and interacting intimately with the intestinal epithelium. The immune dysregulation in Crohn's disease is primarily driven by a Th1 reaction that is initiated by activated dendritic cells. The cross-talk between microbial antigens, IECs, and dendritic cells, studied by Remoldi et al.,[11] suggests the mechanism for homeostasis under normal conditions.

1.1.1 Relevance

With the emerging role of TLR9 in Crohn's, intestinal homeostasis, and probiotic mechanisms of action, the immunological cross-talk must be examined as it occurs between the intestinal epithelium, and innate and adaptive immune cells under basal conditions and inflammation. Thus, with a better understanding of the environmental and immunological interactions in IBD, a complicated disease, we may have a clearer idea about the mechanisms of action of probiotic bacteria DNA used in treatment.

1.1.2 Hypothesis

In a strain-specific and intestinal epithelial dependant manner, bacterial DNA will regulate downstream dendritic and T-cell functional activity.

1.1.3 Scope

The aim of this thesis is to investigate and characterize the role of TLR9 species-specific signaling by the intestinal epithelium through the modulation of innate and adaptive immune functions in normal and inflammatory conditions. Furthermore, this involves the investigation of:

1) TLR9-dependant signaling between bacterial species DNA by IECs,
2) TLR9-activated IECs differentially modulating DC and T-cell activity, and
3) Oral bacteria DNA treatment modulating the immune responses, under basal and inflammatory conditions.

1.2 Genetics of IBD

The involvement of a genetic factor in IBD has been known for decades. Observations of familial clustering combined with concordance rates for Crohn's and UC in monozygotic twins of 35% and 7%, and in dizygotic twins of 11% and 3%, respectively, has revealed the presence of genetic influences in the disease etiology.[16-17] Nevertheless, not until the discovery of the first IBD-associated gene variant, by two separate research groups (Ogura et al.[18] and Hugot et al.[19]) has the direct role of a gene been demonstrated in IBD. The first IBD-associated gene, NOD2/CARD15, discovered in 2001, was found to reside in the IBD1 locus. NOD2/CARD15 transcribes for an intracellular innate immune receptor that recognizes the bacterial cell wall component muramyl dipeptide (MDP). MDP is a peptidoglycan constituent of both gram positive and gram

negative bacteria and results in the activation of the NFκB inflammatory signaling cascade. The mutation of NOD2/CARD15 leads to an altered response to MDP ligand *in vitro*.[18] Nevertheless, NOD2-deficient mice and mice with the NOD2 variants do not spontaneously develop IBD-like phenotypes; and further, no enhanced susceptibility was found to chemical-induced colitis. This highlights the requirement for a multi-allelic deficiency in humans and validates the multifactorial etiology for IBD patients with this mutation.[20] NOD2 is implicated in innate and adaptive immune regulation,[21] and carriers of the variant have decrease IL-10 transcription,[22] describing a functional loss in patients in response to innate immune recognition.

Innate immunity plays a large role in maintaining gut homeostasis. Specifically, Toll-like receptors 2, 4, and 9 are associated with maintaining homeostasis. In a meta-analysis for TLR9 polymorphisms 1237C were found to be associated with Crohn's patients worldwide.[23] These results illustrate the role of innate immune signaling in IBD. Table 1-1(pg 9) illustrates the role of innate immune signaling in IBD with 11 innate immune genes out of the known 30+ genes with IBD susceptibility.[5] Genome-wide association studies (GWAS) have uncovered many of the genes that may not have been considered using the older candidate gene approach. GWAS involves genotyping hundreds of thousands of markers and comparing allele frequencies between large groups of patients and controls. With the advent of high through-put technology, combined with the completed human genome project, and the international HapMap project, there have been great advancements in identification of target genes or

loci associated with IBD patients.[24] GWAS has another advantage in that it is an unbiased search and can help in generating hypotheses, as the results can reveal new candidate genes and pathways yet to be explored in IBD research. GWAS can also validate current known associations in large populations, making it a powerful research tool for an area of research that is understood as involving a multi-genetic background. As of 2008, 30 Crohn's-associated genes and loci had been identified, [5] many with functions related to innate and adaptive immunity, and immune cell signaling. Many of these were described previously using animal models of IBD. These findings are not surprising, as IBD was hypothesized to be partly due to an immune dysregulation to commensal microflora, but they further validate the roles of immune regulation and the microbiota in disease pathogenesis. The identification of immune associated genes in both UC and Crohn's (i.e., STAT3, IL-12p40 and IL-23R) illustrate a common genetic and immunological relationship between these different inflammatory diseases.[25]

Table 1-1 (pg 9) displays the immune-related genetic risk factors for IBD. Most were discovered with GWAS while others used the candidate gene approach and were validated with GWAS. Of the 30 described genetic risk factors in Table 1-1(pg 9), 12 are directly related to known disease effectors in animal and *in vitro* models. They are listed on the basis of function, pattern recognition receptors (PRR), chemokine and cytokines and their receptors, and cellular signaling, all of which could contribute in some way to disease pathogenesis. Briefly, NOD2 mutations result in decreased IL-10

transcription.[26] TLR2, 4, and 9 are associated with maintaining gut homeostasis, [27-28] though by different methods. CCR6 and its ligand CCR20 are required for immune cell homing and lymphoid arrangement, specifically related to mucosal immunity.[29] IFNγ and IL-10 are long known to be associated with Crohn's. Unfortunately, anti-IFNγ has had limited effect in treatment of disease[30]. IL-10 has a distinct role in IBD, as the IL-$10^{-/-}$ mouse model of colitis has been used for decades. The single gene transgenic mutation results in a chronic enterocolitis.[31] IL-12p40 deficiency results in increased susceptibility to infection , but confers a protective effect in chemically-induced colitis.[32] The p40 subunit is part of the IL-23 heterodimer that was recently shown to be associated with the Th17 cell linage and IBD.[33] Also, IL-23R is required for maintaining Th17 cells, which are associated with disease,[34] thus, illustrating the role of IL-23 in disease. The JAK/STAT pathway is involved in cytokine signaling, with IL-6 and IL-10, as well as many other cytokines, that activate STAT3.[35] IL-23R also signals through the STAT3 pathway, showing that STAT3 signaling is involved in important cytokine signaling required for homeostasis. Because these genes play a profound role in host immunology, with many of them being interrelated through intracellular signaling pathways, and because many different mutations can result in the same disease phenotype, the system needed to maintain health is complicated and requires further study. Currently, epistatic studies are being done to determine gene-gene interactions, associated with disease susceptibility. In a multi-allelic disease, where many of the genes have interrelated functions and pathways, these studies can provide

some insight into the combined genetic polymorphisms that may occur in IBD. Functional studies have shown that NOD2 signaling enhances the TLR9-mediated response to CpG DNA, resulting in a synergistic response, which is lost with a NOD2 mutation.[36] Torok et al[37]recently showed that Crohn's carriers of the homozygous or heterozygous NOD2 variant have a higher rate of TLR9-1237T/C polymorphism, compared to controls.[37] Furthermore, when looking at TLR9 polymorphisms, in conjunction with NOD2, IL-23R, and DLG5 allelic variants, the role of TLR9 polymorphisms is apparently in association with Crohn's, but not UC.[37] These studies illustrate the connection between multiple genetic associations found in IBD patients and the role of TLR9 signaling in maintaining gut homeostasis. Thus, a study of the mechanism by which TLR9 mediates gut homeostasis is imperative for understanding and finding new treatments for IBD. As the genetic background plays a large role in disease susceptibility, so too, does the environment, in the form of luminal microflora, and the immune response to it.

Gene symbol	Gene name	Crohn's	UC	Ref.
	Pattern Recognition Receptor			
NOD2	Nucleotide-binding oligomerization-domain-containing 2	Yes	No	[19],[18],[38],[38]
TLR2	Toll-like receptor 2	Yes	No	[23]
TLR4	Toll-like receptor4	Yes	No	[39],[23]
TLR9	Toll-like receptor 9	Yes	No	[23]
	Cytokines, Chemokines and their receptors			
CCR6b	Chemokine (C–C motif) receptor 6	Yes	No	[5],[38]
IFNγ	Interferon gamma	NT	Yes	[38]
IL-10	Interleukin 10	NT	Yes	
IL-12B	Interleukin 12B, IL12p40	Yes	Yes	[5],[38]
IL-23R	Interleukin 23 receptor	Yes	Yes	[5],[40],[41]
	Immune Signaling Cascades			
JAK2b	Janus kinase 2	Yes	No	[5],[38]
STAT3b	Signal transducer and activator of transcription 3	Yes	Yes	[5, 42]

NT= Not tested

Adapted from Barrett et al.[5]

Table 1-1Immune-Related Genetic Risk Factors Associated with IBD

1.3 Microflora and IBD

The adult human body contains approximately 10^{12} cells, and hosts more than 10 times that number of bacteria on the skin and in the gastrointestinal (GI) tract. In both cell number and amount of genetic material, humans are more microbe than men. Humans require gut microbes for normal function, for structure of the mucosal immune system, and for nutrition. Thus, the interaction between the body and the microbes it hosts is imperative for survival. Due to its multiple functions and the requirement of intestinal microflora for health, the intestinal microflora have been suggested to act as a human organ that has evolved closely with us over time.[43] The human GI Tract, from the duodenum to the end of the colon, contains a high antigenic load of approximately 10^{15} bacteria,[44] with approximately 400-1000 different species. The number of species is likely a low estimation[45] due to deficiencies in current methods of analysis. The intestinal microflora and their metabolites are required for human health and play important roles in disease.

IBD is at least partially the result of unregulated immunologic insult against the commensal microflora. The intestine is a highly regulated lymphoid organ with an abundance of mucosal immune cells in close proximity to a huge number of antigenic microflora. The intestinal epithelium is a single cell layer that separates these two highly active organs, and which, under normal conditions, maintains a relative tolerance for each. Because of their close proximity to each other and the large bacterial load, any dysregulation can lead to an exacerbated inflammatory response. Thus, knowledge of the normal microbial content needs

to be combined with our understanding of mechanisms of epithelial tolerance in the study of IBD etiology. Tolerance by the host is maintained by three main immune mechanisms: proper innate immune recognition, proper effecter T-cell activation and response to antigenic stimuli, and immune regulation. Transgenic mouse models of colitis, each having a deficiency in at least one immunologic mechanism (Table 1-2, pg 15); have helped to establish the role of these mechanisms in the pathogenesis of IBD. Moreover, the role of microbiota in disease is being revealed, since most transgenic animal models of colitis have no disease under germ-free conditions. For example the IL-$10^{-/-}$,[46] TGF$\beta^{-/-}$,[47] IL-$2^{-/-}$,[48] TCR$\alpha^{-/-}$,[49] CD45RB^{hi} SCID,[50] Samp1/Yit,[51] and HLA-B27^{transgenic} [52] animal models all result in abrogated disease under germ-free conditions. A more complete list of mouse models, their immune dysregulation, and the role of microflora is shown in Table 2 (pg 15, adapted from Elson et al.'s review).[53] While bacteria play well-known roles in IBD mouse models, they are not involved in all models, and chemically-induced models of colitis can still exacerbate the disease under germ-free conditions.[54] The discrepancy between models having a different immune dysregulation and the role of microflora highlight the need for further clinical research. The clinical evidence is mostly observational, as transgenic human subjects or humans raised in germ-free conditions are not possible. In any case, evidence supports the role of the microflora in disease, and UC and Crohn's occur most frequently in areas of the gut that have the highest bacterial load, the ileum and the colon.[55] Additional evidence also suggests antibiotics may be useful in UC and Crohn's.[56-57] Positive results

have been obtained from fecal diversion studies, with return of disease after fecal stream restoration.[58-60] All of this evidence suggests a strong role for the microbiota in the pathogenicity of IBD, and further studies of the normal roles of the microbiota in health may help elucidate their role in intestinal diseases.

Currently, three, non-mutually exclusive hypotheses describe the role of the microbiota in disease: 1) like gastric ulcers, a not yet identified infectious organism causes disease. 2) Due to a thinner mucus barrier and decreased secretion of defensin, the protection against shearing damage and endogenous microflora is ineffective, which leads to an increase in mucosal-associated bacteria that initiates disease.[61] 3) There is a loss of commensal organisms, which results in a reduced microbial diversity, and gut dysbiosis, and initiating disease. All three of these hypotheses are supported by evidence. Bacteria from the *Mycobacterium*[62] genus and adherent *E. coli*[63] have been suggested as infectious pathogens in IBD. Both types of bacteria have a higher incidence in IBD patients compared to controls, and in some patients, antibodies against bacterial components of these species have been found in IBD patients and may serve as markers for disease.[64] Due to the inherent difficulty in studying the intestinal microflora, the hunt for a single causative infectious microbe has only revealed broad associations so far.

Since the mucus layer in patients with UC is thinner than that of control patients,[65] an increased access to the underlying epithelium is possible. In patients with Crohn's, the composition of the mucus layer is little changed, though a decrease in the anti-microbial peptides, defensins, has been

detected.[66] In addition, an increase in the adherent bacteria was found in patients with IBD.[67] Both of these deficiencies could cause a decreased ability of the gut to protect the host from intestinal microflora and could lead to an increase in the adherent bacteria and disease. Nevertheless, these findings are not known to be causative for the disease. In studying the third hypothesis, inherent complications make any conclusions general, at best. The huge bacterial load and biodiversity, combined with the present inability to culture most of the microbes, leaves researchers with an incomplete picture. While molecular methods of analysis have increased our knowledge of gut components, these are still small snapshots of a larger picture. New methods, based on DNA (i.e., denaturing gel electrophoresis, DGGE), may give us information about the dominant microbes, but information about their amounts and the dynamics of their composition, and whether or not they are viable, may still be undetermined. For example, studies with 16s ribosomal RNA, in combination with metabolomics can give a more detailed picture of the population dynamics and the roles of microbes in the intestine.[68] Other, new culture-independent molecular techniques can offer the greatest insight into the intestinal composition, and allow for the normal intestinal microbiota to be compared to that of patients with inflammatory disorders. To date, 16s ribosomal RNA libraries and metagenomic analyses have shown that, besides bacteria in the gut lumen, large groups of yeast[69] and fungi[70] are also present. Thus, with regards to the third hypothesis, the bacterial biodiversity in patients with Crohn's has become more simplified due to decreases in commensal *Bifidobacteria*[71] and the family

Firmicutes (specifically, *Clostridia* cluster IV[72]), even though our understanding of fungal diversity has increased.[70] The increased fungal community may be due to a microbial imbalance in disease, or due to fungal infections that might play a role in disease, as yet, unexplored possibilities. Regardless of the disease etiology, a greater understanding of the composition of the intestinal lumen will undoubtedly enhance the finding of new treatment options for patients. With mounting evidence for the role of the microbiota in human intestinal disease, and the indisputable role of the microbiota in animal models of IBD, microbial research is likely to be more important in understanding and potentially treating disease.

	Altered Immune Mechanism					
Model	**Innate**	**Effector T-cell**	**Regulatory**	**Microbial role**	**Main defect**	**Ref**
Multi drug resistant gene 1a $^{-/-}$	Y			Maybe - Antibiotics treats	Altered epithelial barrier	[73]
Gα1DD$^{-/-}$	Y			Not Known	Defective Epithelial barrier, defective regulatory B-cell	[74]
Macrophage - PMN Stat3 $^{-/-}$	Y			Maybe	Increased LPS response, resistant to IL-10 regulation	[75]
Bone marrow Stat3 $^{-/-}$	Y			Maybe	Increased response to LPS; impairment of Innate immune function	[76]
DSS	Y			Possible - both + and - studies	Direct damage to epithelium, CD11c+ DC's have role	[54, 77-78]
A20 $^{-/-}$	Y	Y		Not Known	increased response to LPS Has multi organ inflammation	[79]
IL-10 $^{-/-}$	Y		Y	Yes, germ free, and Antibiotics treats	lacks Tr1 activity and TGFβ signaling	[31, 80]
NFκB,DIN$^{-/-}$ p65 $^{+/-}$	Y			Not Known	Increased IL-12 production	[81]
TGFβ $^{-/-}$	Y			Yes	decreased Treg Has multi organ inflammation	[82]
C3H/HejBir	Y	Y		Yes	Impaired response to TLR ligands increased bacterial responsive T-cells	[83]
SAMP1/YIT	Y	Y		Yes	Epithelial cell defect, increased activated T-cells	[51], [52]
CD4+, CD45RB^{hi}	Y		Y	Yes	decreased Treg Has multi organ inflammation	[50]
IL-2 $^{-/-}$			Y	Yes	Decreased CD4+, CD25+ population	[48, 84]
IL-2 receptor αL$^{-/-}$			Y	Maybe	Decreased CD4+, CD25+ population	[85]
TCR $\alpha^{-/-}$			Y	Yes	Loss of regulatory B cells	[49], [86]
TGFβGFDI$^{-/-}$	Y		Y	Not Known	Decrease Treg Has multi organ inflammation	[87]
Smad3 $^{-/-}$			Y	Not Known	Decrease Treg Has multi organ inflammation	[88]
Stat4 ^{transgenic}		Y		Maybe	Excessive bacterial specific effecter T-cell response	[89]
CD40L ^{transgenic}		Y		Not Known	Increased activated T-cell Has multi organ Inflammation	[90]
TNFαN$^{\Delta NFD}$		Y		Not Known	Increased TNFα production Has multi organ Inflammation	[91]

Adapted from Elson et al.[53]

Table1-2: Mouse Models of IBD: Immune Mechanism and Role of Bacteria

1.4 Adaptive Immunity and IBD

The adaptive immune system is required to protect us from recurring infections (memory), and to induce tolerance toward self antigens (regulation). Nevertheless, abnormalities or deficiencies in any arm of the adaptive immune system can lead to inflammatory disorders, such as IBD, and autoimmune disorders (i.e., immunodysregulation polyendocrinopathy enteropathy X-linked, IPEX; caused by a lack of FOXp3 Tregs).[92] The adaptive immune system consists of both T- and B-cells, though IBD is associated with T-cell disorders only. Current mouse models of colitis are the result of immune deficiencies in three main areas: innate immune signaling, effector T-cell activity, and regulation (Table 1-2 pg 15). The T-cell subtypes that are associated with IBD are: Th1, Th2, Th17, and T-reg. Specifically, Crohn's is associated with an overactive adaptive Th1/Th17 immune response, with cytokines IL-6, IL-12/23, IFNγ, TNFα, and the classical inflammatory cascade implicated in the pathology.[93] Patients with Crohn's have higher amounts of Th1 commensal-specific effector cells in non inflammed biopsies, and in *ex vivo* explants. Such biopsies and explants produce significantly more cytokines than those of control patients, when activated with heat-killed commensal organisms. UC is associated with a Th2-predominant disease, and cytokines (i.e., IL-4, IL-5, and IL-13) are implicated with the disease pathology.[94] UC does not have a classical Th2-type reaction, because, like Crohn's, IFNγ and TNFα have also been detected.[95] The role of the IL-23/Th17 axis in UC is less studied than in Crohn's, but IL-23R polymorphisms have been found to be associated with disease susceptibility in

both diseases (Table 1-1 pg 9). Higher amounts of IL-23 RNA and IL-17 production from lamina propria $CD4^+$ T-cells have been detected in both Crohn's and UC, suggesting a role in disease.[96] Moreover, the role of the IL-23/Th17 axis in IBD has not been fully elucidated, but the $IL\text{-}10^{-/-}$ mouse model of colitis, when back-crossed with $IL\text{-}23p19^{-/-}$ mice, were disease-free at 12 months of age, illustrating the delicate balance required to maintain gut homeostasis.[97] As cytokines, secreted by the T-helper cell lineages, play a role in innate and adaptive immunity, and variations of their classically-defined cytokine profiles have been observed in IBD, IBD is suggested to be partially due to an imbalance in effector cell populations combined with decreased regulation.[4]

The role of adaptive immunity and IBD is best illustrated with the multiple T-cell transfer models of disease. $CD4^+CD45RB^{hi}$ cells, transferred into a syngeneic immune-compromised host (i.e., SCID or $Rag^{-/-}$), results in colitis within 6-12 weeks of transfer.[98] Furthermore, the transfer of $CD4^+CD45RB^{low}$ cells, containing the population of $FOXp3^+$ Tregs, fully abrogates colitis. The ability to cure disease illustrates the potency of suppressor T-cells in immune regulation and homeostasis. While the role of $FOXp3^+$ Tregs in human disease is less clear than in disease models, UC and Crohn's have been reported to have fewer Treg cells in inflamed biopsies, compared to that found in other intestinal inflammatory disorders.[92] In any case, the number of suppressor T-cells is higher than that seen in non inflammed control patients. This suggests a potential lack, but not a complete loss, of Tregs with IBD-associated inflammation. The role of Tregs in maintaining gut homeostasis is partially understood, but how they

exert their function remains unclear. Some reports indicate that soluble factors IL-10 and TGF-β are involved, since $CD4^+CD45RB^{low}$ cells from either knockout are unable to completely abrogate disease.[99],[100] While, *in vivo* data suggests that the mechanism is through intimate cell-cell contact and co-receptors, Tregs in the lamina propria and MLN can be in contact with T-effector cells as well as APCs. T-egs, missing chemokine receptors CCR4 and CCR7, which function to home to the lamina propria, are unable to suppress colitis, to suggest that they need to be in the same discrete region as the T-effector cells. Further evidence for intimate interaction is illustrated in $CD4^+CD45RB^{hi}$ recipient mice without CD103 co-receptors on DCs, where Tregs transfer is unable to suppress disease. Nevertheless, this is not seen with *in vitro* models.[92] Thus, the mechanism of action is not yet fully understood, but Tregs are probably mediating their activity with a combination of secreted soluble factors and cell-cell contact.

APCs, like DCs are bridges between adaptive and innate immunity. They can be activated by both innate immune receptors and through a classical presentation of protein antigen on MHC Classes I and II to activate $CD8^+$ and $CD4^+$ T-cells. In the intestine, a heterogeneous pool of DCs plays a vital role in mucosal immunity by initiating inflammatory response to pathogenic bacteria and maintaining homeostasis. DCs sample the luminal content by extending, thin cellular projections, dendrites, through the tight junctions of epithelial cells. DC's are located below specialized M-cells in the Peyer's patches of the small

intestine to initiate rapid immune responses. The role of DCs in maintaining gut homeostasis and disease is rigorously studied but not fully understood.

Memory $CD4^+$ Th1 cells, reactive to intestinal microflora, have been described in the pathology of disease.[101],[102] DCs are required to present bacterial-specific antigens to T-cells andare able to initiate a memory response. Under normal conditions, DCs are able to prime the $CD4^+$ Th1 response to commensal bacteria. Indeed, this activity depends on the MHCII presentation of antigen.[103] The authors suggest that a lack of regulation of Th1 effector cells may be occurring in IBD patients. The population of $CD4^+$ commensal-reactive T-cells may be enlarged in IBD, possibly due to the increased permeability of the gut and a greater interaction between luminal bacteria and the mucosal DCs. In addition, the C3H/HejBir[102] and the SCID[104] transfer model of colitis have highly active commensal-specific $CD4^+$ T-cells, while controls do not. Furthermore, increased populations of inflammatory associated DC's that produce IL-12- and IL-18 are found in inflamed tissues of CD patients suggesting their role in disease.[105]

Like the role of genetics and microbiota, the role of the adaptive immune system in the pathology of IBD is still unclear. Distinct alterations have been found in the T-effector cells of the Th1, Th17, Th2, and Treg subsets, associated with disease, and the most abundant APCs found in the mucosal immune system, DCs, have been implicated in IBD, either through priming of an abnormal T-effector cell response to commensal microflora, an inflammatory response,[106] or by mediating a loss of regulation.[107] In any case,

complicated mechanisms appear to be involved in maintaining gut homeostasis and the role of the adaptive immune system in the pathology of IBD.

1.5 Innate Immunity and IBD

The innate immune system is an evolutionarily conserved system maintained to protect the host from infections and disease. Its primary roles are to initiate an immediate response to foreign organisms and initiate processes that will lead to an adaptive immune response, if necessary.[108] The specificity of innate immunity is rudimentary, compared to the adaptive immune system; however, it recognizes a large repertoire of conserved molecular motifs associated with many infectious organisms. The innate immune system consists of pattern recognition receptors (PRR) that detect pathogen-associated molecular patterns (PAMPs) though not all recognized PAMPs are from pathogens. Of the innate immune receptors NOD-like receptors (NLRs) and Toll-Like Receptors (TLRs) are associated with IBD.[109] The most studied PRR to date are the TLRs, named after the Toll receptors in drosophila that protect against fungal infections in the host.[110] Human TLRs currently have eleven known receptors that recognize cell surface and intracellular components of bacteria, viruses, and parasites. They recognize a variety of moieties including carbohydrates, nucleic acids, lipopeptides, and bacterial structures such as flagellin.[111] Since the discovery of the first TLR[112], the pathway and functions of TLRs in maintaining gut homeostasis and IBD has been studied. TLRs are found on many cell types, though most are immune cells such as antigen-presenting cells, T-cells, and mucosal surfaces. TLRs 1-9 are found in

both small and large intestinal epithelial cells, as shown by the presence of either RNA or protein.[111] Intestinal epithelial cells (IECs) are in direct contact with the luminal microbes, which contains many, if not all, of the PAMPs that are recognized by the PRRs. IECs are no longer considered to be a static barrier that separates an environment overloaded with antigenic stimuli from the active immune system. IECs, in response to TLR stimulation, secrete immune-active cytokines and chemokines to induce the migration and activation of immune cells.[14, 113-114] IEC's are able to condition adaptive immune cells, like DCs, to induce specific T-cell effector responses.[113-115] Innate immune receptors on IECs prime the mucosal immune system to maintain a tolerance to the vast amount of antigenic stimuli, while maintaining the ability to induce an appropriate immune response to pathogenic bacteria.[116] The exact mechanism and the role of TLRs has become a focus of IBD research, as evidence has been found that polymorphisms in TLRs 2, 4, and 9 are associated with disease (Table 1-1 pg 9). The induction of TLRs by their ligands leads to initiation of an inflammatory response, mainly through NFκB, through pathway crossover other transcription factors, such as AP-1 and STATs, may also be activated and have profound immunological effects.[117] This illustrates the complexity by which TLR's function. In healthy individuals, no sustained inflammation is seen, though TLRs are in constant contact with their ligands. The mechanism by which the gut can maintain homeostasis while being able to mount an appropriate response is not completely understood. Nevertheless, evidence indicates that homeostasis is maintained partially by hyporesponsive receptors, compartmentalized receptors,

and combinations of receptors on different cell types that integrate TLR responses.[118-119] DC and IEC TLR2 and 4 DCs, have been shown to be hyporesponsive to their cognate ligands protecting the host from constant inflammatory signals.[120] While other TLRs are compartmentalized, TLR3, 7, and 9, are found in endosomal vesicles in DCs, to isolate them from self nucleotides, and requiring processing by the acidic environment of the vesicles. TLR9 has also been found on the cell surface of IECs and is in constant contact with luminal antigens. In addition, germ-free animals do not demonstrate TLR9 surface staining.[13] These observations suggest that TLRs have a role in normal gut physiology in response to bacteria DNA. Lee et al.[28] showed a differential response to TLR9-signaling in polarized IECs, which depended on apical or basolateral treatment, giving further evidence to support the compartmentalization hypothesis, with luminal activation resulting in a tolerant response to maintain homeostasis, where basolateral activation results in an inflammatory response.[28] Interestingly, mice deficient in the main adaptor molecule (MyD88) or TLR4 or TLR9, are more susceptible to chemical-induced colitis, to further suggest a protective role of these molecules in maintaining homeostasis. Previously, Madsen and colleagues have shown that IECs respond differentially to DNA isolated from probiotic and pathogenic bacteria.[13-14] The results suggest that commensal signaling is protective, yet IECs are able to mount an inflammatory signal to pathogens. The full mechanism by which IECs differentiate, or the extent to which this differentiation occurs, is not understood. The aim of this thesis is to shed light on the differential response and to test

whether or not it can be communicated to adaptive immune cells so as to modulate their function.

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Chapter 2. Experimental Models

2.1 HT29 Cells

HT-29 cells are mature human colonic epithelial cells that were originally obtained from a female patient with colon carcinoma in 1964. These immortalized cells form confluent monolayers and, when grown on semi-permeable membranes (Transwell®), allows separate access to each surface of the monolayer. HT-29 cells secrete a distinct array of pro-inflammatory cytokines (IL-8, MCP-1, GM-CSF, and TNF□) on exposure to invasive bacteria or pro-inflammatory mediators. HT-29 cells express several innate immune receptors, including toll-like receptors (TLRs) 2-9. These cells respond to the TLR4 ligand, LPS, with activation of both MAP kinase and NF-κB and IL-8 secretion [1-2]. However, HT-29 cells are poorly responsive to TLR2 ligands as determined by production of IL-8. [3] Expression of TLRs 4, 5, 7, and 9 have been shown to change in response to their specific ligands.[4] Previous work has demonstrated that HT-29 cells respond to bacterial DNA in a differential manner; that is, DNA from probiotic bacteria such as *Bifidobacteria* did not induce an IL-8 response while DNA from a pathogen such as *Salmonella* does induce. Further, it was shown that DNA isolated from the probiotic VSL#3 resulted in an attenuation of IL-8 secretion from HT-29 cells in response to TNFα or *Salmonella*, and a delayed NF-κB signaling. This delay in NF-κB signaling was due to a TLR-9 induced inhibition of proteasome activity and a decreased

degradation of the IκB subunit.[2] These findings laid the groundwork for the experiments performed as part of this thesis.

2.2 MODE-K Mouse Duodenal Epithelial Cell Line

MODE-K mouse duodenal epithelial cells were immortalized by a SV40 T cell gene using a murine ectopic virus as a vector in 1993[5]. These cells maintain their polarized phenotype and have a microvillus on the apical membrane. MODE-K cells were derived from a CH3/He female mouse and express H-2^k MHCII molecules. Under basal conditions the expression of MHCII is minimal; however, when stimulated with IFNγ, MODE-K cells express high levels of MHC II [5]. This induction of MHCII expression gives MODE-K cells the ability to induce differentiation in naive bone marrow derived T-cells in MHCII matching mouse bone marrow[6]. MODE-K cells express mRNA for TLRs 2, 4, 9, and 11. In addition, the levels of TLR9 and 11 have been shown to be up-regulated in response to parasitic infection, verifying that they are functional[7]. Further supporting the use of MODE-K cells for the experiments described in this thesis, studies have demonstrated that probiotic bacteria can attenuate a TNFα-induced secretion of the pro-inflammatory chemokine, IP-10. Together, these findings that MODE-K cells respond to bacteria, and can act as antigen-presenting cells; make them a good experimental model system for the experiments laid out in this thesis.

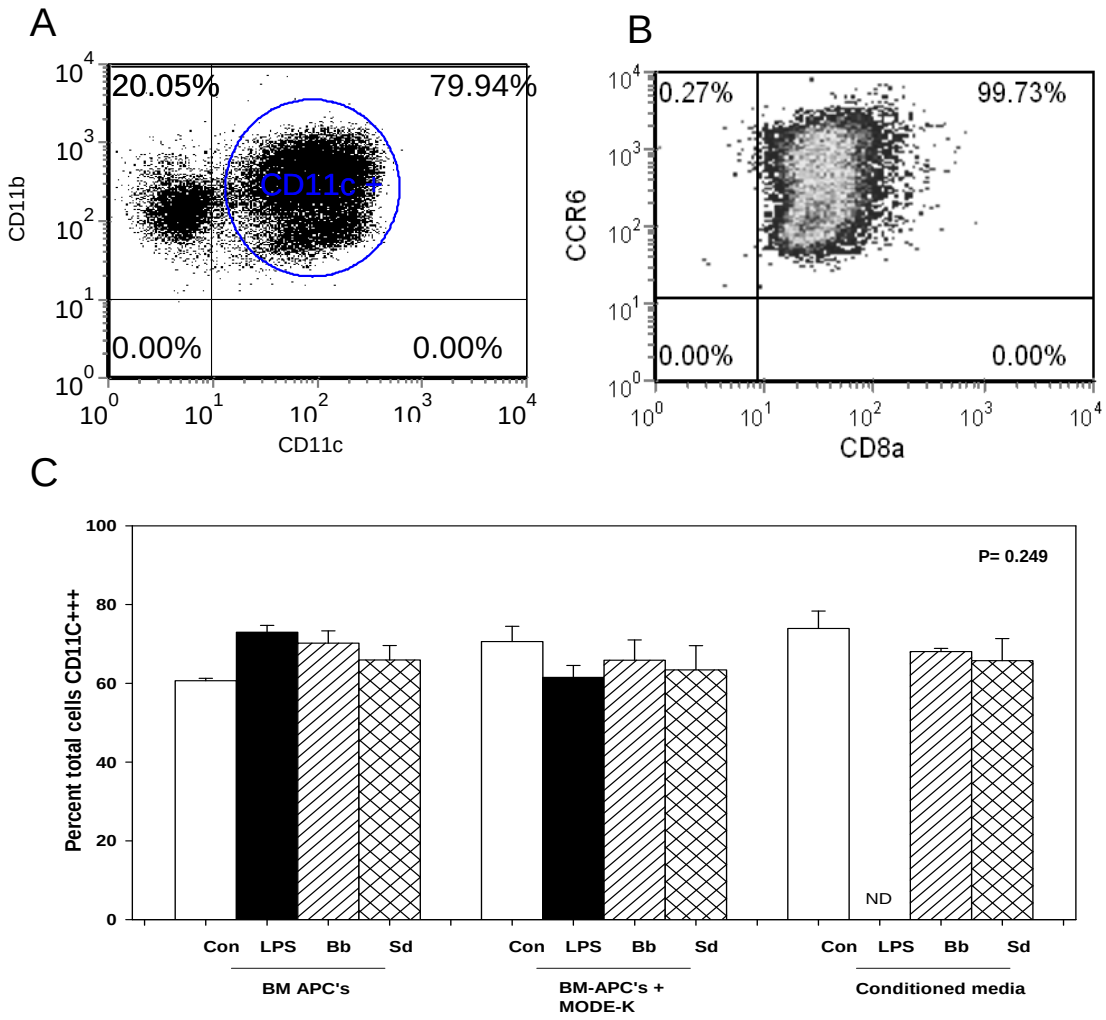
2.3 Bone Marrow-Derived Antigen Presenting Cells

Antigen presenting cells (APC's) are major players in the mucosal immune system and bridge the adaptive and innate immune system. Both dendritic cells and macrophages act as professional APC's in the gut, and function to maintain homeostasis as well as acting to eradicate invasive microorganisms[8]. Together, epithelial cells and antigen-presenting cells modulate and drive the innate and adaptive immune responses to luminal microflora. This relationship between epithelial cells, bacterial DNA, and antigen-presenting cells was explored in the experiments described in this thesis; in essence, a physiological co-culture system was developed whereby epithelial cells were stimulated on their apical surface with bacterial DNA while bone-marrow derived antigen presenting cells and T cells were in the basolateral compartment.

Dendritic cells (DCs) can be defined based upon their functional activity and their extracellular markers. Although the use of dendritic cells isolated from the gut mucosa may be more physiological relevant for these types of experiments, it is extremely difficult to obtain enough cells to perform experiments. In addition, the act of isolating mucosal DCs may in itself change their activity. In the studies described in this thesis, DCs were derived from bone marrow of 129 Sv/Ev mice. Bone marrow derived DCs were cultured using a modified protocol based on the original Inaba protocol[9]. Using the protocol laid out by Lutz et al.[10] using only GM-CSF to differentiate the cells, a mixed population of CD11c+++ (70%) and CD11b+ (30%) cells was obtained, suggesting a mixed population of conventional DCs and macrophages (BM-

APCs) (Figure 2-1A pg 36)[11]. To further differentiate phenotype, the cell surface markers CD8α (a marker for ability to produce IL-12p70) [11] and CCR6 (a chemokine receptor for homing to Peyer's Patches), were measured by flow cytometry. At time 0 and after 24 hrs of culture, all CD11c+ cells were CD11b+, CCR6+, CD8α+ (Fig 2-1B pg 47). Treatment in co- culture or conditioned media for 24 hrs did not affect the cell surface markers or the ratio of cDC's and macrophages (Fig 2-1C pg 47).

To confirm that the isolated population of BM-APCs expressed TLR9 and responded to TLR9 ligands, BM-APCs were incubated with DNA from *Bifidobacterium breve* or DNA from *Salmonella dublin*. Both DNAs induced TLR9 up-regulation as measured by real-time PCR after 2 hours of incubation (Figure 2-2 pg 37). This finding confirmed TLR9 expression and functional response, and allowed us to use these preparations for the experiments described in this thesis.



ND= not determined

Figure 2-1. Composition, phenotype, and activation of BM-APC's as analyzed by flow cytometry.

A) Representative plot of cells $CD11c^{+}$, $CD11b^{+}$,
B) Cells CCR6+, CD8a+
C) Percent cells CD11b+, CD11c+ averaged over all preparations after treatment.

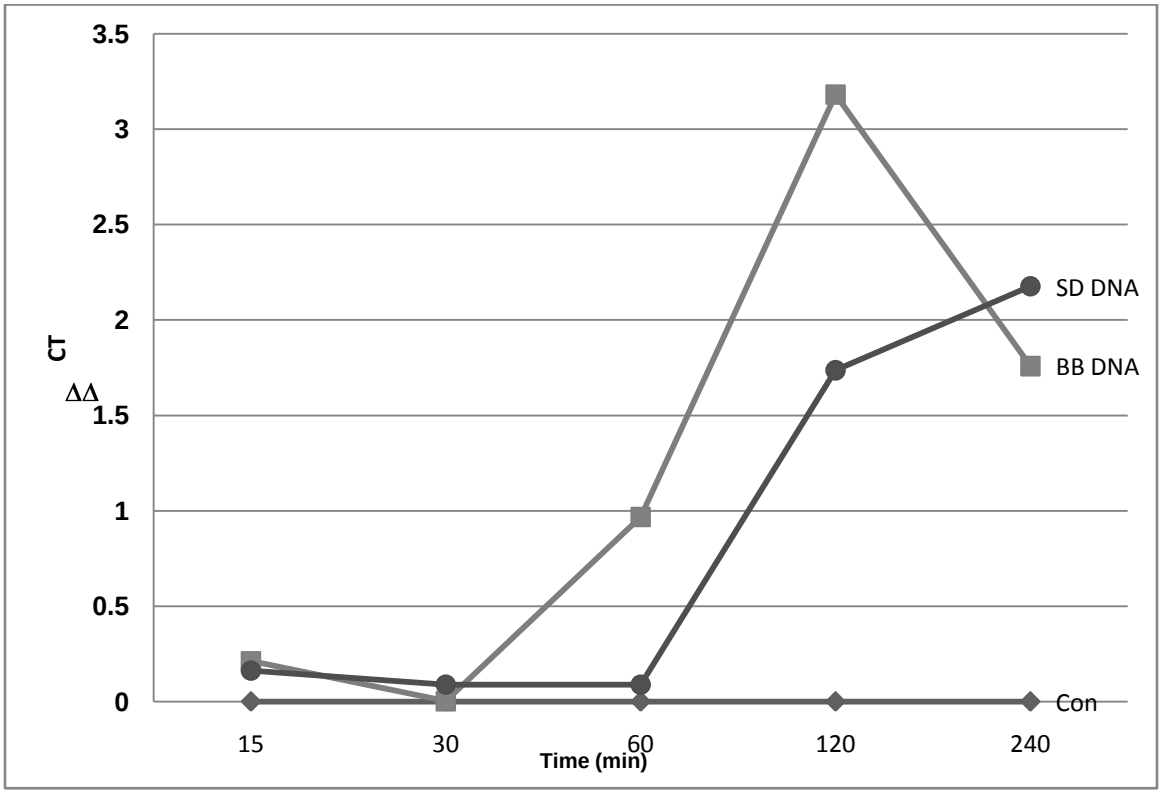


Figure 2-2. Level of TLR9 mRNA in BM-APCs after induction with *Bifidobacteria breve (BB)* and *Salmonella Dublin (SD)* bacterial DNA. TLR9 mRNA levels as detected by real time RT-PCR.

2.4 $CD4^+$ T-cells from the Spleen and Mesenteric Lymph Nodes of 129 Sv/ev mice

CD4+ T-cells are important in combating infections and cancers, and aberrant regulation and effector responses by this group of immune cells is associated with chronic inflammatory conditions such as IBD.[12] Within this group of immune cells are four known subsets that have differential function and distinct cytokine profiles. These subsets have been labeled as Th1, Th2, Th17, and T regulatory cells, based primarily on their cytokine secretion patterns (Figure 2-3 pg 40). Naïve $CD4^+$ T cells are stimulated to differentiate into mature T-cell subsets based upon the patterns of signals they receive from antigen presenting cells and the surrounding cytokine milieu.[13] $CD4^+$ T-cells express co-stimulatory molecules such as CD80/86, and TLR's that modulate their activity.[14] [15] Of the TLR's expressed on $CD4^+$ T-cells, TLR9 can functionally act as a co-stimulatory molecule resulting in increased proliferation and preventing anergy. [16] *In vitro* studies of CD4+ T cells often use cells harvested from the spleen and mesenteric lymph nodes (MLN). In that these organs contain a myriad of immune cells, isolation methods need to be employed to isolate pure CD4+ cultures. Cell sorting by FACS or magnetic bead technology has allowed for 95-98% pure cultures.[17] *In vitro* cultures of CD4+ T-cells are often artificially activated using antibodies against CD28 and CD3, co-stimulatory molecules to enhance their *in vitro* response.[18] In models where there is no protein antigen to be loaded on the MHCII of the BM-APC's, it is imperative that

T-cells are activated in this fashion so as not to artificially induce anergy due to a lack of co-stimulation.

Due to the complexity of the *in vivo* mucosal immune system and the highly antigenic load of the luminal contents, studies performed in this thesis primarily used a reductionist model by culturing individual cell types involved in maintaining gut homeostasis under single and co-culture conditions. Figure 2-4 (pg 41) compares the *in vivo* and *in vitro* systems. BM-APC'S were activated in a transwell culture system by apically stimulating the epithelial monolayer with either LPS or bacterial DNA. After 24 hrs of activation the intestinal epithelial monolayer was removed and CD4+ T-cells isolated from the spleen or MLN of mice were added to the BM-APC culture for 48hrs. Due to the lack of protein antigen in the culture system, the co-culture was supplemented with beads coated with antibodies against CD28 and CD3. This allowed for proper TCR activation and co-stimulation of the CD4+ T-cells. It is through such a reductionist system involving both epithelial and immune cells that the roles of each cell type can be studied both in isolation and together, to help understand how they function under more *in vivo*.

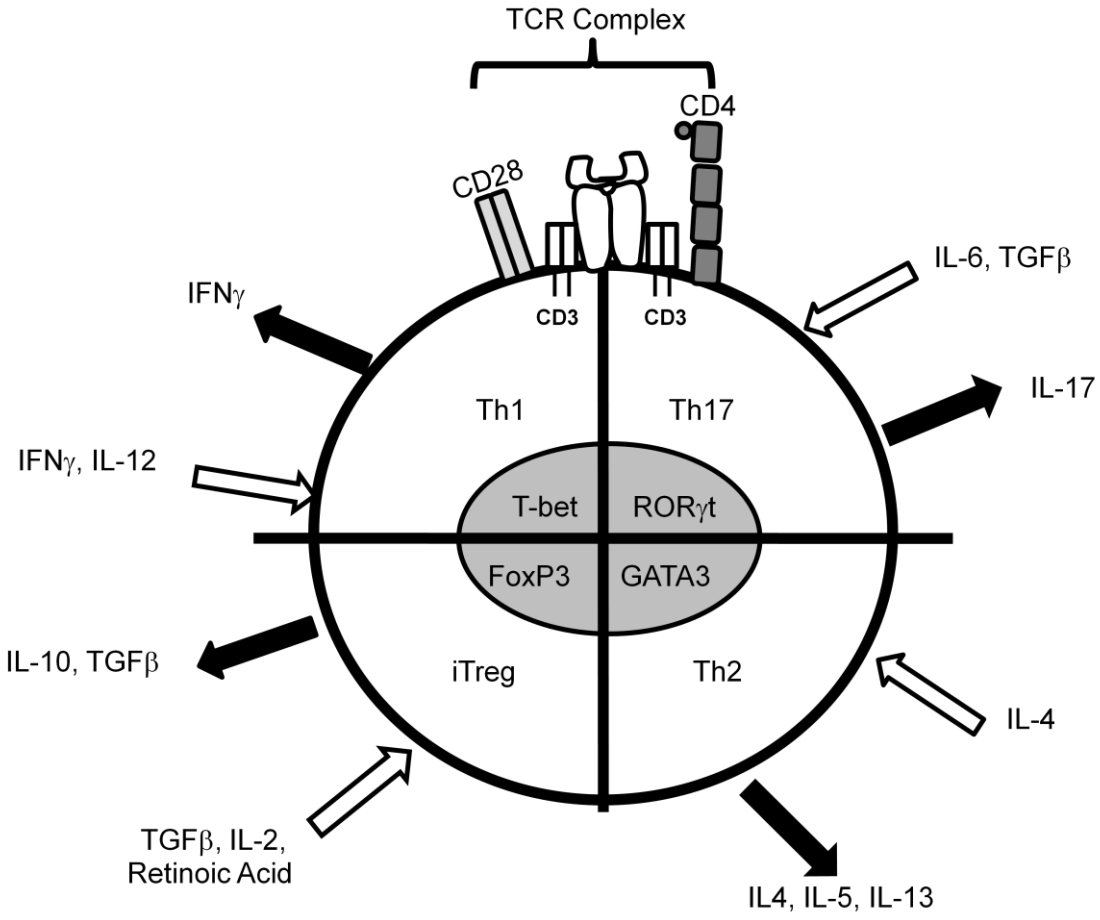


Figure 2-3. CD4^{+} T-Cell Lineages and Primary Cytokines. Upon encountering an antigen presenting cell naïve CD4+ T-cells receive signals from the cytokines in their environment (open arrows) to differentiate into one of four T-helper (Th) cell lineages Th1, Th2, Th17, and iTreg. This leads to activation of specific signaling pathways resulting in nuclear translocation of lineage specific transcription factors (inner circle). Effector Th cells secret cytokines (black arrow) into their environment to recruit and activate other immune cells and modulate an appropriate immune response.

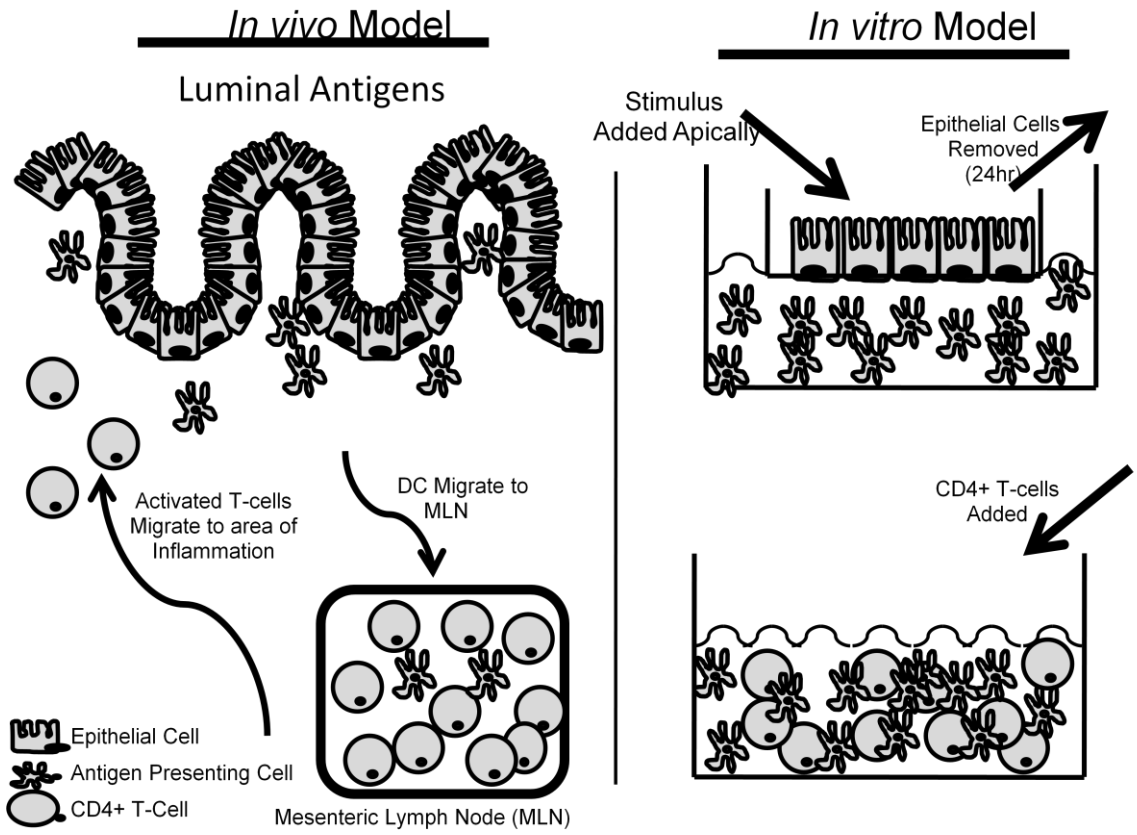


Figure 2-4. Comparison of *in vivo* and *in vitro* systems. In the mucosal immune system, antigen presenting cells underlie the intestinal epithelium and sample from the lumenal contents. Activated APC's migrate to a draining lymph node to activate naïve T-cells. Once activated, these cells migrate back to the area of inflammation and modulate immune response to the invading antigen. The *in vitro* culture model has activation of dendritic cells occurring through a monolayer of intestinal epithelial cells by stimulation of a single TLR (TLR9 by DNA; TLR4 by LPS). After 24 hrs, the epithelial monolayer is removed and naïve CD4+ T-cells are cultured with the APC's for a further 48 hrs.

2.5 129 Sv/Ev IL-10$^{-/-}$ Mouse Model of Colitis

IL-10$^{-/-}$mice spontaneously develop a patchy Crohn's-disease-like colitis that is characterized by focal ulcerations and transmural lesions. The immune dysregulation is initiated by CD4^{+} T cells and consists of an IL-23 and IL-17 directed excessive generation and activation of Th1 cells directed against the commensal microflora. The onset and degree of inflammation are highly dependent upon gut microflora, allowing for the design of experiments directed against modifying the gut microbiota. [19] [20] This model of disease is totally dependent on microbial flora as animals reared under germ free conditions or treated with antibiotic therapies have no signs or attenuated disease respectively.[21-22] Previous studies have demonstrated that these mice respond to probiotic bacterial DNA with an attenuation of inflammation,[23] Further, TLR9 is expressed on surface epithelium much like our cultured IEC's.[24] Thus, this model was chosen to complement the *in vitro* studies in order to investigate the role of epithelial cells in modulating the immune response to TLR9 ligands.[24]

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Chapter 3. Bacterial DNA Induces Differential Gene Expression in Human Intestinal Epithelial cells.

4-1 Introduction

Intestinal epithelial cells (IEC's) are a single cell layer that separates a highly antigenic luminal milieu and a highly organized mucosal immune system. It is their primary role to maintain a tight barrier against luminal bacteria and antigens while allowing absorption of required nutrients and water. Further, IECs play a large role in maintaining gut homeostasis and guiding immunological responses to luminal antigens.[1] The mechanisms by which they do this and their role in diseases, such as IBD are only now being understood.

In epithelial cells, the primary means of recognition of microbes is through innate pattern recognition receptors, including Toll like receptors. IEC's express TLR's 1-9 however the pattern of expression is cell line dependant.[2-3] Toll like receptors recognize conserved molecular patterns found in microbial components including lipopeptides, zymosan, nucleotides, flagellin, and Lipopolysaccharide. HT29 cells express TLR's 1, 2, 3,4,7,5 and 9 as determined by protein expression and RNA.[3-4]

TLR9 signaling is associated with gut homeostasis where loss of TLR9 signaling results in increased susceptibility to DSS induced colitis. In addition, TLR9 is required for the anti-inflammatory affects of probiotics.[5] TLR9 recognizes unmethylated deoxyribo cytosine-phosphate-guanine (CpG) motifs

found in high amounts in bacterial DNA.[6] TLR9, like most TLR's, signals through the Myd88/NFκB signaling cascade culminating in nuclear translocation of NFκB, a central regulator of inflammatory and immune responses.[7] TLR9 is expressed on the epithelial surface, unlike the expression found on immune cells, and is not dependant on endosomal acidification for activation.[8] Further, epithelial TLR9 responds differentially to genomic bacterial DNA by secreting the inflammatory chemokine IL-8.[8] Our lab has previously shown that DNA isolated from probiotic mixture VSL#3 is able to modulate the NFκB signaling cascade due to an inhibition of proteosome function.[9] This mounting evidence suggests a role for IEC specific TLR9 signaling in maintaining gut homeostasis. The aim of these experiments was to use immune specific microarrays to identify functional pathways at a molecular level in IEC that are induced by probiotic and pathogenic bacteria DNA, in order to better understand how epithelial cells differentially respond to bacterial DNA.

3-2 Materials and Methods

3-2-1 Bacterial strains and preparation of DNA

Salmonella enterica serovar dublin strain Lane (ATCC #15480) was selected for these studies as a pathogenic bacteria due to the ability of its DNA to induce IL-8 secretion from intestinal epithelial cells.[9] *Bifidobacteria breve* Y8 (VSL Pharmaceutics) was selected as a probiotic bacteria due to the ability of its DNA to reduce basal IL-8 secretion.[9] Bacteria were inoculated at 0.18% (v/v) into 25 ml of Mann-Rogosa Sharpe broth (Difco #0370-17-3) and grown statically

overnight (18 – 20 h) at 37°C. For DNA isolation, cells were centrifuged at 11700*g* for 10 min, washed with SSC buffer (0.15 NaCl, 0.015 M sodium citrate) and re-suspended in 0.01M sodium phosphate buffer with 20% sucrose and 2.5 mg/ml lysozyme for 45 min at 37°C followed by lysis buffer (10mM Tris-HCl, 1mM EDTA, 500 mg pronase B, 1% SDS, pH 8) for 30 min at 37°C. DNA was extracted by adding an equal volume of 1:1 buffer-saturated phenol and chloroform to the bacterial solution. The mixture was spun for 5 min at 4000 rpm, and the aqueous layer removed. The extraction was repeated until no interface was visible. Traces of phenol were removed with chloroform and the salt concentration adjusted by the addition of 1/10 volume of sodium acetate, pH 5.2. DNA was precipitated with cold 100% ethanol, washed with 70% ethanol and re-suspended in sterile Tris-EDTA buffer. A post isolation treatment to remove endotoxin was performed with a 5% volume of Tx114 (Promega) at 4ºC for 30 min. Further isopropanol precipitation was done to isolate endotoxin-free DNA. Only preparations with endotoxin levels not exceeding 0.1ng endotoxin per µg DNA were used. Concentration and purity of DNA preparations was confirmed by measuring OD_{260} absorbance, $OD_{260/280}$ ratio, and by running agarose gel electrophoresis as previously described [14]. DNA preparations were assayed for endotoxin using the *Pyrochrom* amebocyte assay (DP0704, Cape Cod Inc, MA, and USA). DNase treated preparations were used in all experiments as control preparations. DNA preparations were incubated overnight at 37°C with 5 mg/ml DNase 1 (Sigma-Aldrich) in the presence of 5mM $MgCl_2$. DNA depletion was confirmed by agarose gel electrophoresis with ethidium bromide staining as

previously shown [8]. Prior to cell culture treatment, DNase was heat-inactivated at 90ºC for 10 min.

3-2-2 HT29 Human Small Intestinal Cells

HT-29 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 (Gibco, Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal calf serum (Cansera, Rexdale, ON, Canada). All experiments were conducted in serum-free media. Cells were seeded at a density of 1 x 10^6 cells/well and were maintained at 37°C in a 5% CO_2 atmosphere. Fresh medium was added to cultures daily. Cells were grown in 12-well tissue culture plates (Falcon, New Jersey) for Taqman low density array. Cells were treated with either *Salmonella dublin* (SD) DNA (50 μg/ml), *Bifidobacterium breve* (BB) DNA (50 μg/ml), TNFα (10ng/ml) for 2 hrs and isolated on ice using Trizol (Gibco, Burlington, ON, Canada).

3-2-3 Real-time Taqman Low Density Array

Total RNA was isolated with Trizol (Gibco) by following the manufacturer's instructions. Quality and quantity of RNA was measured using a Nanodrop ND-100 spectrophotometer (Nanodrop, Wilmington, DE, USA). mRNA was reverse transcribed using high capacity cDNA reverse transcription kit following manufacturer's instructions. A real-time Taqman low density array analysis was performed with an ABI 7900 sequence detector (Applied Biosystems, Branchburg, NJ). The reaction mixture (total volume, 100ul) consisted of 1ug cDNA with double-distilled H_2O to a volume of 50ul, and 50ul Taqman Universal

PCR Master Mix (2×) (Applied Biosystems). Samples were loaded onto a Taqman low density Human immune array panel (Applied Biosystems). Panels were centrifuged, sealed and run following manufacturer's instructions. Analysis of relative quantity was done using RQ manager 1.2 (Applied Biosystems).

3-2-4 Statistical Analysis

Data were tested for normality of distribution and analyses performed using the statistical software SigmaStat (Jandel Corporation, San Rafael, CA). Differences between means were evaluated using analysis of variance, student *t*-tests, or by a nonparametric Mann-Whitney Rank Sum test where appropriate. Specific differences were tested using the Dunn's or Bonferroni post hoc analysis.

3-3Results

3-3-1 HT29 IEC's Respond Differentially to Bacteria DNA

To examine IEC gene expression in response to probiotic and pathogenic bacteria DNA, HT-29 cells were treated with DNA from *Salmonella dublin* (SD) or *Bifidobacteria breve* (BB) and cells harvested at 15, 20, 60, and 120min. TNFα was used as a positive control. RNA from the cells was isolated and reverse transcribed. An immune specific Taqman low density human array panel was run and analyzed. The 120min time point was chosen for further analysis due to the increased differences between treatments. Relative quantity (RQ) was determined using a time zero untreated control group. Endothelin converting enzyme 1 (ECE1) was chosen as an endogenous control as it had the tightest

clustering of amplification curves compared to the other possible endogenous controls (Figure 3-1 pg 52). 18sRNA was used as a loading control. The Taqman low density human immune panel (TLDA) has 96 genes hits in duplicate from varying relevant immune pathways (Appendix 1). As seen in Table 3-1 (pg 51), BB treatment induced 52 genes with 47 genes resulting in a greater than 2-fold difference between SD DNA treatments, while SD DNA and TNFα induced only 32 and 33 genes respectively. In addition, SD and TNFα down-regulated 25 and 29 genes, while BB DNA down-regulated 13 genes. These results clearly demonstrate that epithelial cells respond to probiotic and pathogenic bacterial DNA with different patterns of gene expression. The genes were grouped into 5 functional groups; 1) cytokines, chemokines and secreted factors; 2) cellular markers; 3) intracellular signaling molecules; 4) apoptosis; and 5) enzymes and are discussed below

	BB DNA			SD DNA			TNFα		
	Up reg	Down reg	ND	Up reg	Down reg	ND	Up reg	Down reg	ND
Cytokines	11	2	7	8	4	8	9	4	7
Chemokines	9	2	3	8	3	3	8	4	2
Secreted factors	1	1	2	0	2	2	0	2	2
cellular markers	9	0	9	3	4	11	5	4	9
Intracellular signaling	4	1	2	3	2	2	2	3	2
Apoptosis	3	1	1	1	1	3	0	1	4
Enzymes	5	2	0	3	4	0	2	5	0
Degranulation, compliment	3	0	1	1	1	2	2	1	1
cellular migration	5	0	2	3	0	4	3	1	3
endogenous controls	2	4	0	2	4	0	2	4	0
Total number of genes	**52**	**13**	**27**	**32**	**25**	**35**	**33**	**29**	**30**

ND=Not detected

Table 3-1 Summary of Gene Expression in HT29 cells Treated with Bacteria DNA

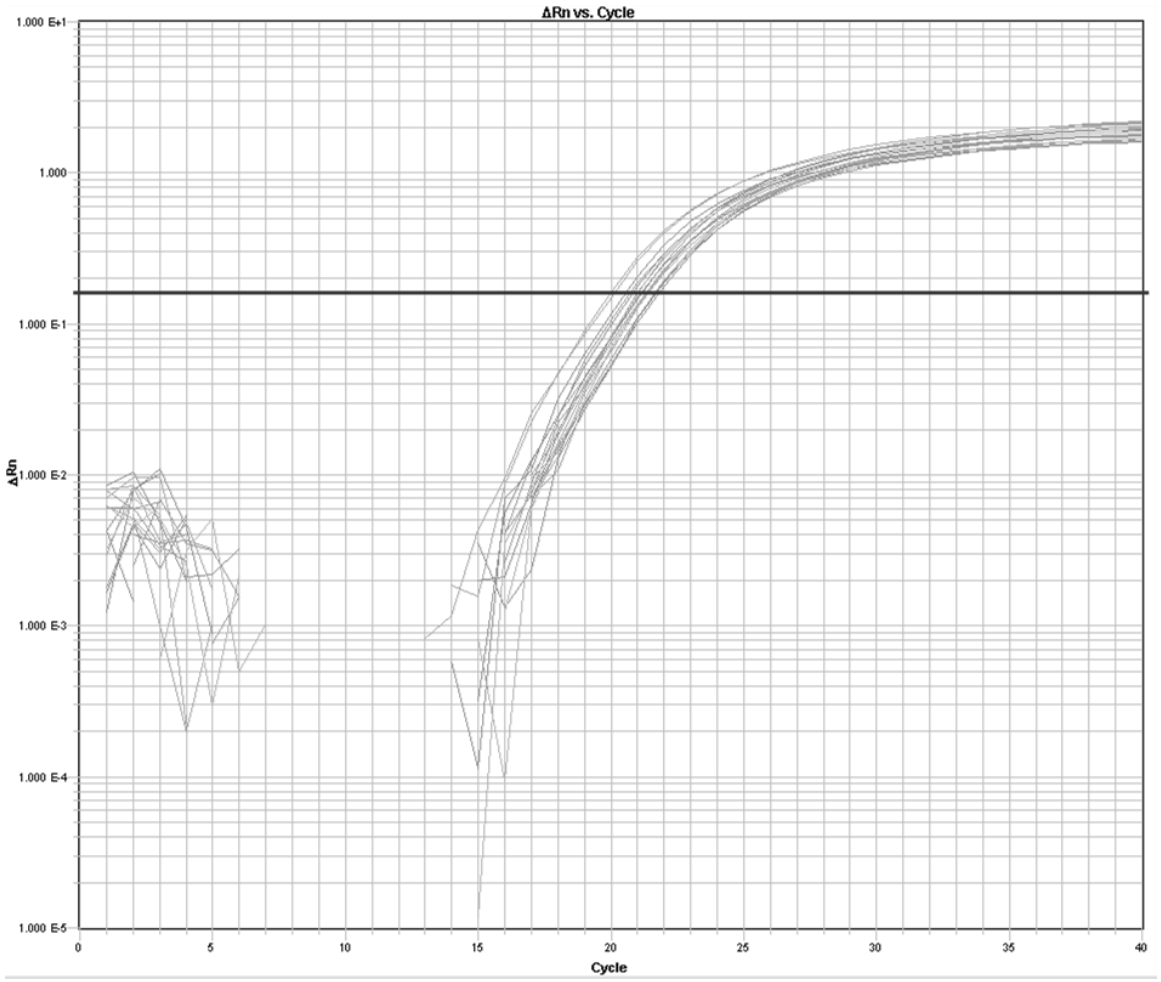

Figure 3-1 Amplification plot of Endogenous control ECE1

3-3-2 Cytokines and Chemokines

Cytokines and chemokines are the messages that control multi-cellular immune responses. The TLDA panel used in these experiments analyzed the expression of 38 different pro and anti inflammatory cytokines and chemokines. As seen in Table 3-2(pg 58), BB DNA up-regulated 21 and down-regulated 5 separate genes while SD DNA resulted in only 16 up-regulated and 9 down-regulated respectively. There was no amplification detected for IL-2, IL-3, IL-10, IL-12β, IL-17, CCR2, CCR5, IFNγ, iCOS for any of the treatments. IL-4 was not detected with SD DNA treatment but was up-regulated by BB DNA. Of the 25 genes in this group where amplification was detected, 16 of the genes had greater than a 2 fold difference between SD and BB DNA treatment (Table 3-2 pg 55). This is congruent with work done in our lab that has shown differential IL-8 response in a strain specific manner to bacterial DNA.[9]

These differential messages could potentially play roles in downstream $CD4^+$ T-cell differentiation as the environmental cytokine milieu help to differentiate naïve T-cells into effector sub populations.[10] IL-4, IL5 and IL13 associated with the Th2 inflammatory cascade where IL-4 is required for the differentiation into Th2 T-cells. BB DNA but not SD DNA, induced IL-4 mRNA message. Further, BB DNA also induced IL-5 whereas SD DNA resulted in decreased message. Crohn's disease is a Th1 mediated disease, and induction of a Th2 message by probiotic bacterial DNA may be helpful by returning the immunological balance in Th1 and Th2 cells, that is associated with homeostasis. However, interestingly, BB DNA also induced IL-6 and IL-18 above those levels

induced by SD DNA. Both IL-6 and IL-18 have pro-inflammatory actions, inducing Th17 cells and IFNγ secretion, These findings were suggest a potential role for the induction of T helper cell phenotypes, further investigation into the ability of IEC's to modulate adaptive immune function in response to bacterial DNA is an aim of this thesis.

		Log$_{10}$RQ			Fold difference
Cytokines					
Gene symbol	**Gene name**	**BB DNA**	**SD DNA**	**TNFα**	**SD Vs BB**
IL-1A	Interleukin 1 a	-0.18	0.01	0.77	-1.90
IL-1B	Interleukin 1 b	0.563	0.205	0.64	-3.58
IL-2	Interleukin 2	**ND**	**ND**	**ND**	**ND**
IL-3	Interleukin 3	**ND**	**ND**	**ND**	**ND**
IL-4	Interleukin 4	1.14	**ND**	2.38	**ND**
IL-5	Interleukin 5	0.62	-0.79	-1.54	-14.10
IL-6	Interleukin 6	1.57	0.34	-0.19	-12.30
IL-7	Interleukin 7	0.28	0.09	-1.37	1.90
IL-8	Interleukin 8	0.7	0.51	2.56	1.90
IL-9	Interleukin 9	**ND**	**ND**	**ND**	**ND**
IL-10	Interleukin 10	**ND**	**ND**	**ND**	**ND**
IL-12A	Interleukin 12p35	0.07	-0.13	0.64	-2.00
IL-12B	Interleukin 12p40	**ND**	**ND**	**ND**	**ND**
IL-13	Interleukin 13	-1.09	0.02	0.24	11.10
IL-15	Interleukin 15	1.51	-0.31	0.74	-18.20
IL-17	Interleukin 17	**ND**	**ND**	**ND**	**ND**
IL-18	Interleukin 18	2.24	0.2	-0.2	-20.40
IFNG	Interferon gamma	**ND**	**ND**	**ND**	**ND**
TNF	Tumor Necrosis Factor	0.91	0.36	3.24	-5.50
TGFβ1	transforming growth factor-beta 1	0.11	-0.13	0.07	-2.40
CSF1	colony stimulating Factor 1	0.8	0.32	1.55	-4.80
CSF2	colony stimulating Factor 2	1.55	0.42	1.47	-11.30
CSF3	colony stimulating Factor 3	**ND**	**ND**	2.21	**ND**
LTA	Lymphotoxin alpha (TNF superfamily, member 1)	**ND**	**ND**	**ND**	**ND**
Chemokines					
Gene symbol	**Gene name**	**BB DNA**	**SD DNA**	**TNFα**	**SD Vs BB**
CCL3	Chemokine (C-C motif) ligand 3	1.62	1.35	1.15	-2.70
CCL19	Chemokine (C-C motif) ligand 19	-2.7	-0.69	-0.07	20.10
CCL2	Chemokine (C-C motif) ligand 2	2.91	-0.39	2.39	-33.00
CCL5	Chemokine (C-C motif) ligand 5	-0.03	0.1	-0.58	1.30
CCR2	chemokine (C-C motif) receptor 2	**ND**	**ND**	**ND**	**ND**
CCR4	chemokine (C-C motif) receptor 4	0.6	-0.07	-0.6	-6.70
CCR5	chemokine (C-C motif) receptor 5	**ND**	**ND**	**ND**	**ND**
CCR7	chemokine (C-C motif) receptor 7	0.819	0.523	0.619	-2.96
CXCR3	chemokine (C-C motif) receptor 3	1.22	0.15	0.03	-10.70
CXCL10	Chemokine (C-X-C motif) ligand 10	0.63	0.15	0.58	-4.80
CXCL11	Chemokine (C-X-C motif) ligand 11	0.09	0.14	-0.32	0.50
Secreted Factors					
Gene symbol	**Gene name**	**BB DNA**	**SD DNA**	**TNFα**	**SD Vs BB**
ICOS	inducible T-cell co- stimulator	**ND**	**ND**	**ND**	**ND**
NOS2A	nitric oxide synthase 2A	0.1	-0.11	-0.28	-2.10
VEGF	vascular endothelial growth factor A	-0.07	-0.02	-0.29	0.50

ND- Not detected

Table 3-2. Relative quantity of immune genes induced by bacterial DNA in HT29 cells. Values are expressed in log10 at 120min following bacterial exposure and normalized to time zero.

3-3-3 Cellular Markers

It has been suggested that due to the ability of IEC's to constitutively express MHCII[11] and to secrete peptide loaded MHCII molecule in exosome like vesicles,[12] that IEC's act as specialized antigen presenting cells (APC's) producing "tolerosomes" to maintain homeostasis.[11, 13] When gene expression of cellular surface markers was analyzed, there was no detected amplification of either MHCII molecule HLA-DRA or HLA-DRB1 in response to BB or SD DNA. . This suggests that HT29 IEC's do not constitutively express MHCII message as suggested by Lin et al.[11] Further, levels of co-stimulatory molecules such as CD80, CD86, also were not detected. This suggests that under conditions in these experiments, HT-29 cells did not express a functional MHCII complex or act as an APC. However, message for macrophage markers, CD68, ICAM-1, and the T-cell marker CD28 were detected. These molecules were previously detected in healthy human biopsies and suggest a role for IEC's in immune modulation.[11] Treatment with BB DNA resulted in up-regulation of message in all three of these genes as well as the T-cell markers CD8α, CD4, CD38. SD DNA induced, to a lesser extent, ICAM1, CD38, CD8α, CD68 while CD4 and CD28 were down regulated (Table 3-3 pg 58). This further illustrates the differential cellular response to bacterial DNA. These results suggest that IEC's treated with BB DNA may have a greater ability to intimately interact with APC's due to the expression of higher T-cell markers CD4, CD8α, and co-stimulatory marker CD28. Due to the inability to interact with APC's with a fully functional TCR, the single co-stimulatory message from CD28 and CD4 may

result in anergy of the antigen presenting cell. Thus, BB DNA may induce non-active APC's where SD DNA treated APC's would not have this ability. Further work would need to be done to look at protein expression and intimate contact with IEC's and APC's to determine if this is a mechanism in which probiotics act to modulate the inflammatory response.

		Log$_{10}$RQ			Fold difference
Cellular markers					
Gene symbol	**Gene name**	**BB DNA**	**SD DNA**	**TNF α**	**SD Vs BB**
CD3E	Cluster of differentiation 3E	**ND**	**ND**	**ND**	
CD4	Cluster of differentiation 4	0.03	-0.03	-0.82	-0.60
CD8A	Cluster of differentiation 8a	1.26	0.84	0.37	-4.20
CD19	Cluster of differentiation 19	**ND**	**ND**	**ND**	**ND**
CD12RA	The interleukin 2 (IL-2) receptor alpha	**ND**	**ND**	1.53	**ND**
CD28	Cluster of differentiation 28	0.52	-1.34	-0.44	-18.61
CD34	Cluster of Differentiation factor 34	**ND**	**ND**	0.31	**ND**
CD38	Cluster of differentiation 38	1.39	0.27	-0.27	-11.20
PTPRC	protein tyrosine phosphatase, receptor type, C	4.06	**ND**	**ND**	**ND**
CD68	Cluster of differentiation 68	0.17	0.04	-0.46	-1.30
CD80	Cluster of differentiation 80	2.55	**ND**	**ND**	**ND**
CD40LG	Cluster of differentiation 40 ligand	**ND**	**ND**	**ND**	**ND**
HLA-DRA	major histocompatibility complex, class II, DR alpha.	**ND**	**ND**	**ND**	**ND**
HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	**ND**	**ND**	**ND**	**ND**
CTLA4	Cytotoxic T-Lymphocyte Antigen 4	**ND**	**ND**	**ND**	**ND**
TNFRSF18	tumor necrosis factor receptor superfamily, member 18.	1.08	-0.01	0.26	-10.90
TBX21	T-box 21	**ND**	**ND**	**ND**	**ND**
SKI	v-ski sarcoma viral oncogene homolog	0.59	-0.09	0.17	-6.80

ND- not detected

Table 3-3. Relative quantity of immune genes induced by bacterial DNA in HT29 cells Values are expressed in log10 at 120min following bacterial exposure and normalized to time zero.

3-3-4 Intracellular Signaling Genes

There were several differences in HT-29 cell intracellular signaling responses to BB compared with SD DNA. In comparison with SD DNA treatment BB DNA induced greater levels of the transcription factors STAT3, NF-κB, IκBκB, and SMAD3 (Table 3-4 pg 61). STAT3 is a potent transcription factor that positively regulates IL-10 and IL-6 transcription. Further, STAT3 is associated with IBD susceptibility[14] and plays a role in a variety of biological pathways including cell growth, regulatory cytokine production, and apoptosis.[15] STAT3 signaling in innate immune cells and colonic epithelial cells has been shown to be beneficial by maintaining epithelial barrier function cellular viability.[15] The induction in STAT3 by probiotic derived DNA may then be enhancing this protective mechanism.

NFκB is well known as a potent immune regulator and is also involved in cellular proliferation, differentiation and apoptosis,[16] both BB and SD DNA induce NFκB mRNA expression, suggesting both DNA signal through this pathway. Surprisingly, BB DNA induced NFκB at a three-fold higher level. The induction of NFκB by BB DNA would seem to be incongruous with data that shows probiotic DNA inhibits NFκB translocation.[9] BB DNA also induced the inhibitor of NFκB inhibitor IKBKB (Table 3-4 pg 61) while SD DNA inhibited expression. This further suggests an increased role for NFκB in early BB DNA treatment. NFκB is a master regulator of many relevant immunological cascades and is involved in initiating transcription of many of the genes on this panel. As

we see increased transduction of genes regulated by NFkB in early BB DNA treatment it's not surprising to see increased mRNA for their transcription factor. Probiotic derived DNA has been shown to inhibit NFκB activity by inhibiting the proteosome.[9] This was detected 30 min after 2hr incubation with the isolated DNA. The discrepancy between our mRNA data and the data previously reported may be due to timing or the cells are producing increased NFκB and IκBκB message but the active unit is not translocating to the nucleus but due to proteosomal inhibition. Further work would need to be done to validate this observation and to determine the level of translocation and activation by NFκB itself.

3-3-5 Apoptosis Genes

Apoptosis plays many vital roles in regulating immune responses including regulating effector cell populations, cancer cell growth, and maintaining a healthy epithelial barrier.[17] The human immune array has gene targets that include both the intrinsic and extrinsic apoptotic pathways. BB DNA induced the anti-apoptotic gene BCL2L1 and pro apoptotic BAX from the intrinsic pathway and up-regulated FAS message from the extrinsic pathway, while SD DNA down regulated BCL2L1 and BAX and only mildly increases FAS. Both DNA down-regulated FASL, suggesting the induction of a survival signal by the epithelial cells (Table 3-4 pg 61).

		Log_{10} RQ			Fold Difference
Intracellular signaling					
Gene symbol	**Gene name**	**BB DNA**	**SD DNA**	**TNFα**	**SD Vs BB**
STAT3	signal transducer and activator of transcription 3	0.59	0.02	-0.11	-5.70
NFKB2	Nuclear factor Kappa B p49	0.46	0.09	0.93	-3.70
IKBKB	Inhibitor of nuclear factor kappa B kinase beta subunit	0.35	-0.01	-0.35	-3.60
SMAD3	SMAD, mothers against DPP homolog 3	0.72	0.03	0.51	-6.90
SMAD7	SMAD, mothers against DPP homolog 7	-0.11	-0.02	-0.75	0.90
AGTR1	angiotensin II receptor, type 1	**ND**	**ND**	**ND**	**ND**
AGTR2	angiotensin II receptor, type 2	**ND**	**ND**	**ND**	**ND**
Apoptosis					
Gene symbol	**Gene name**	**BB DNA**	**SD DNA**	**TNFα**	**SD Vs BB**
BCL2	B-cell CLL/lymphoma 2	**ND**	**ND**	**ND**	**ND**
BCL2L1	BCL2-like 1	0.313	-0.14	-0.53	-4.53
BAX	BCL2-associated X protein	0.25	-0.07	-0.38	-3.20
FAS	Tumor necrosis factor receptor superfamily member 6	0.19	0.07	-0.44	-1.20
FASLG	Fas ligand (TNF superfamily, member 6)	-0.29	-0.25	-0.76	0.40
Enzymes					
Gene symbol	**Gene name**	**BB DNA**	**SD DNA**	**TNFα**	**SD Vs BB**
HMOX1	heme oxygenase1	0.89	-0.04	-0.5	-9.30
PTGS2	Prostaglandin-endoperoxide synthase 2	0.343	0.00	1.14	-3.42
RPL3L	Ribosomal protein L3-like	1.08	0.05	-0.33	-10.29
CYP1A2	Cytochrome P450, family 1 , subfamily A, polypeptide 2.	-2.70	-0.92	-0.19	17.78
CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1	-0.10	0.38	0.67	4.77
REN	renin	0.22	-0.06	-0.79	-2.80
ACE	angiotensin I converting enzyme	0.13	-0.12	-0.23	-2.48

ND- not detected

Table 3-4 Relative quantity of immune genes induced by bacterial DNA in HT29 cells. Values are expressed in log10 at 120min following bacterial exposure and normalized to time zero.

		Log_{10} RQ			Fold Difference
Degranulation, Complement					
Gene symbol	**Gene name**	**BB DNA**	**SD DNA**	**TNFα**	**SD Vs BB**
PRF1	perforin 1	0.59	0.19	-0.39	-4.1
GZMB	granzyme B	2.17	**ND**	0.74	**ND**
GNLY	granulysin.	**ND**	**ND**	**ND**	**ND**
C3	complement component 3	0.07	-0.29	1.15	-3.6
Cellular Migration					
Gene symbol	**Gene name**	**BB DNA**	**SD DNA**	**TNFα**	**SD Vs BB**
FN1	fibronectin 1	0.59	0.00	-0.15	-5.9
COL4A5	collagen, type IV, alpha 5	**ND**	**ND**	**ND**	ND
LRP2	Low density lipoprotein- related protein 2	1.63	1.40	**ND**	-2.3
ICAM1	inter-Cellular Adhesion Molecule 1	0.83	0.07	2.27	-7.6
SELP	selectin P	1.84	1.75	1.83	-0.9
SELE	selectin E	1.95	**ND**	1.97	
Possible Endogenous Controls					
Gene symbol	**Gene name**	**BB DNA**	**SD DNA**	**TNFα**	**SD Vs BB**
MYH6	myosin, heavy chain 6	**ND**	**ND**	**ND**	**ND**
EDN1	endothelin 1	-0.29	-0.26	-0.76	0.3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase.	2.28	0.06	0.36	-22.2
ACTB	actin, beta	2.59	-0.10	0.49	-26.8
TFRC	transferrin receptor	6.25	2.32	3.42	-39.3
GUSB	glucuronidase, beta	0.21	-0.06	-0.41	-2.6
PGK1	phosphoglycerate kinase 1	0.73	-0.22	-0.71	-9.5
ECE1	Endothelin converting enzyme	END	END	END	ND

Table 3-5 Relative quantity of immune genes induced by bacterial DNA in HT29 cells. Values are expressed in log10 at 120min following bacterial exposure and normalized to time zero.

3-4 Discussion

In this study we have shown that gene expression in epithelial cells in response to bacterial DNA is species specific. Surprisingly, overall probiotic DNA induced a greater number of genes than did DNA from pathogenic bacteria DNA, while SD DNA treatment down regulated a greater number of genes than did BB DNA. While a functional differential cytokine and chemokine response from IEC's in response to different bacterial species has been shown previously, here we demonstrate that the differential gene response extends to numerous signaling pathways and transcription factors. The differential secretion of cytokines by IEC upon stimulation with bacterial DNA would be expected to alter the environment that underlying immune cells (macrophages, dendritic cells, CD4+ T cells) would be exposed to in the gut. In that the cytokine milieu that naïve $CD4^+$ T-cells are exposed to is very important in determining their differentiation into specific effector subsets, this modulation of IEC gene expression and cytokine secretion would certainly have down-stream effects on immune cell stimulation in the gut. (Figure 2-3 pg 40).

3-5 Limitations of Data

This data presented in this section is preliminary and needs to be tested further with biological replicates and gene validation. Much of the hypothesis generating approaches such as microarrays require these further steps to determine not only significance but reproducibility. What we can take away from

this data is purely observational where we see that HT29 intestinal epithelial cells respond in a very different way to DNA Isolated from two bacterial species.

3-6 Further Discussion

This data clearly shows that there is a differential response to bacterial DNA in a species specific way. How intestinal epithelial cells are able to differentiate between bacteria DNA in a species specific manner is currently unknown. It may partially due to the cellular localization of TLR9on IEC's. APC's and adaptive immune cells like T-cells TLR9 is located in the endosomal vessicle. The endosomal environment facilitates DNA degradation and is required for TLR9 activation in these cell types. In direct contrast to adaptive immune cells, intestinal epithelial cells express TLR9 on both the apical and the basolateral membranes.[8, 18] Further, depending on the surface that the cell senses immune-stimulatory CpG motifs there is a differential response that has been associated with colonic homeostasis.[18] The apical membrane is in constant contact with lumen antigens including microbial DNA and the basolateral membrane is in direct contact with the lamina propria and the mucosla immune system. This surface should only see bacterial antigens if the barrier in breached. Thus, it would be during infection that TLR9 would see antigen under these conditions an inflammatory response would be necessary to initiate a proper immune response. It is unknown how cellular localization modulates the immune response to TLR9 but highly interesting. How TLR9 is able to differentiate between bacterial species is also unknown. There is evidence to suggest that it is sequence specific where bacterial motifs of 5'

purines, unmethylated CpG, and then two 3’ pyrimidines as the most stimulatory and TTAGGG motifs act as self DNA and have inhibitory properties.[19] Methylation decreases the stimulatory nature of DNA.[8] Determining if there are different amounts of stimulatory to non stimulatory motifs or the level of methylation in bacterial DNA may have an effect on its ability to activate TLR9. There are TLR9 independent DNA sensing systems. These innate immune receptors are found in the cytosolic fraction of cells, activate the type one interferon cascade, and respond to different DNA motifs than TLR9. Currently the cellular expression is unknown.[20] To determine if there is collaboration between the TLR9 dependant and independent DNA sensing systems, which may have an impact on the differential response to bacterial DNA, TLR9 knockdown studies using siRNA should be done.

These data confirm that intestinal epithelial cells act in a differential manner in response to species specific bacteria DNA. The remainder of this thesis will focus on how interactions of epithelial cells with bacterial DNA mediate underlying immune cell functional activity.

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Chapter 4 Bacterial DNA differentially induces IL-17 producing T cell responses through interactions with intestinal epithelial and dendritic cells

4-1 Introduction

The apical surface of the intestinal epithelium is in close contact with luminal microbes and their products, while the basolateral surface is closely associated with immune cells of the underlying gut-associated lymphoid tissue. Recognition of luminal microbes and the ability to discriminate between harmful and commensal organisms is a seminal role of the innate immune system. Recent studies suggest that epithelial cells in collusion with dendritic cells and macrophages act together to orchestrate appropriate mucosal innate immune responses and maintain gut homeostatsis.[1-2] It has been shown that epithelial cells release soluble factors that condition underlying dendritic cells to prime $CD4^+$ T helper cells to undergo type-2 (Th2) differentiation and secrete IL-4 and IL-10 in response to live and irradiated bacteria.[2-3] In addition, Rimoldi et al [2] have shown that gut dendritic cells are conditioned by factors derived from epithelial cells to become “noninflammatory”. These dendritic cells are unable to release IL-12 and activate inflammatory Th1 cells highlighting the role of epithelial cells in maintaining homeostasis.

Epithelial-microbial interactions generally involve innate immune receptors such as toll-like receptors (TLRs).[4] TLRs play critical roles in early innate recognition and inflammatory responses against pathogenic microbes. Gut

epithelial cells express TLR1-TLR9, as well as the nucleotide-binding oligomerization domain (NOD) molecules, Nod1 and Nod2.[5] TLR9 is activated by unmethylated CpG containing DNA found in high concentrations in microbial DNA, some double-stranded DNA viruses, and synthetic CpG-oligodeoxynucleotides (ODN)[4, 6]. In epithelial cells, TLR9 is expressed on the cell surface, and is up-regulated in response to the presence of pathogenic bacteria DNA.[7-8] The epithelial response to bacterial DNA is dependent upon whether TLR9 is activated on the apical or basolateral surface, and also the specific bacterial strain.[7, 9] TLR9 signaling is associated with gut homeostasis, where loss of TLR9 signaling results in increased susceptibility to DSS induced colitis. Further, TLR9 is required for the anti inflammatory affects of probiotics,[10-11] demonstrating a role for probiotic bacteria DNA as an active factor that imparts a beneficial effect. More recently, Hall et al [12] have shown a role for TLR9 signaling and gut flora DNA in modulating the levels of T regulatory and T effector cells in the gut. Based on these findings, we hypothesized that the differential response of epithelial cells to probiotic and pathogenic bacterial DNA would be communicated through dendritic cell signaling and result in altered T cell differentiation. Here we demonstrate that treatment of epithelial cells with pathogenic bacterial DNA results in a suppression of IL-12p70 secretion concomitantly with an increase in IL-6, IL-1β, and IL-10 from underlying bone-marrow derived dendritic cells and a subsequent enhancement of IL-17 secretion from CD4+ T cells. In contrast, probiotic bacterial DNA treatment of epithelial cells, while also suppressing IL-12p70

secretion, did not result in any increase in cytokine secretion from bone-marrow derived dendritic cells or elicit any IL-17 secretion from CD4+ cells. These findings highlight the role of TLR9 and epithelial cells in modulating gut innate immune responses through dendritic cell and CD4+ T cell responses, and provide a mechanism by which the intestinal epithelium can induce tolerance to commensal organisms while providing a robust immune response to pathogens.

4-2 Materials and Methods

4-2-1 Bacterial strains and preparation of DNA

Salmonella enterica serovar dublin strain Lane (ATCC #15480) was selected for these studies as a pathogenic bacteria due to the ability of its DNA to induce IL-8 secretion from intestinal epithelial cells.[13] *Bifidobacteria breve* Y8 (VSL Pharmaceutics) was selected as a probiotic bacteria due to the ability of its DNA to reduce basal IL-8 secretion.[13] Bacteria were inoculated at 0.18% (v/v) into 25 ml of Mann-Rogosa Sharpe broth (Difco #0370-17-3) and grown statically overnight (18 – 20 h) at 37°C. For DNA isolation, cells were centrifuged at 11700*g* for 10 min, washed with SSC buffer (0.15 NaCl, 0.015 M sodium citrate) and re-suspended in 0.01M sodium phosphate buffer with 20% sucrose and 2.5 mg/ml lysozyme for 45 min at 37°C followed by lysis buffer (10mM Tris-HCl, 1mM EDTA, 500 mg pronase B, 1% SDS, pH 8) for 30 min at 37°C. DNA was extracted by adding an equal volume of 1:1 buffer-saturated phenol and chloroform to the bacterial solution. The mixture was spun for 5 min at 4000 rpm, and the aqueous layer removed. The extraction was repeated until no interface

was visible. Traces of phenol were removed with chloroform and the salt concentration adjusted by the addition of 1/10 volume of sodium acetate, pH 5.2. DNA was precipitated with cold 100% ethanol, washed with 70% ethanol and re-suspended in sterile Tris-EDTA buffer. A post isolation treatment to remove endotoxin was performed with a 5% volume of Tx114 (Promega) at 4ºC for 30 min. Further isopropanol precipitation was done to isolate endotoxin-free DNA. Only preparations with endotoxin levels not exceeding 0.1ng endotoxin per µg DNA were used. Concentration and purity of DNA preparations was confirmed by measuring OD_{260} absorbance, $OD_{260/280}$ ratio, and by running agarose gel electrophoresis as previously described [14]. DNA preparations were assayed for endotoxin using the *Pyrochrom* amebocyte assay (DP0704, Cape Cod Inc, MA, and USA). DNase treated preparations were used in all experiments as control preparations. DNA preparations were incubated overnight at 37°C with 5 mg/ml DNase 1 (Sigma-Aldrich) in the presence of 5mM $MgCl_2$. DNA depletion was confirmed by agarose gel electrophoresis with ethidium bromide staining as previously shown [8]. Prior to cell culture treatment, DNase was heat-inactivated at 90ºC for 10 min.

4-2-2 Bone Marrow- Derived Antigen Presenting Cell Isolation

APC's cells were isolated from the long bones of male 129Sv/ev mice using a modified protocol [14]. Briefly, long bones of mice were isolated and cleaned, then placed in 70% ETOH for 5 min and transferred to calcium and magnesium-free PBS (CMF-PBS). The ends of bones were clipped and the marrow rinsed with 10ml CMF-PBS. Cells were spun @ 2000 RPM for 5 min,

re-suspended in CMF-PBS and plated at 2 x 10^6 in 10 ml media. Cells were maintained at 37° C with 5% CO_2 in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, penicillin (1000 units/ml), streptomycin (1μg/ml), L-glutamine (2mM) (Invitrogen) and 1000U/ml mouse recombinant GM-CSF (R&D). After 10 days of incubation, the cellular mixture was stained with $CD11c^+$, $CD11b^+$ and 7ADD to determine viability. Following, incubation, two primary groups of cells were identified by flow cytometry. The majority of the cells (~80%) were CD11b+ and CD11c+, while ~20% of cells were CD11b+ and CD11c- (Figure 2-1 pg 36) and were thus defined as bone-marrow derived antigen presenting cells (BM-APCs). On day 10, the cells were counted and plated at a concentration of 1x10^6 cells/ml without mrGM-CSF. BM-APC's were then incubated for 24 hrs with either Lipopolysaccharide (*E. coli* LPS: 1 μg/ml), *Salmonella dublin* (SD) DNA (50 μg/ml), *Bifidobacterium breve* (BB) DNA (50 μg/ml), or conditioned media derived from MODE-K cells (CM). Following incubation, flow cytometry analysis demonstrated that all cells were positive for CD11c, CD11b, CCR6, and CD8α and showed up-regulation of surface MHCII IA/IE marker (Figure 2-1 pg 36).

4-2-3 MODE-K Mouse Small Intestinal Cells

Mouse intestinal cells (MODE-K) immortalized with mouse SV40 large T gene transfer with mouse ectopic virus exhibit similar morphological and phenotypic characteristics of normal enterocytes [15] and were used for these studies. The cells were a generous gift from Dr. K Croitoru (University of Toronto, Toronto, ON). The cells were maintained at 37º C and 5% CO_2 in DMEM high

glucose with HEPES and L-glutamine (Invitrogen) supplemented with 10% heat inactivated FBS. To prepare conditioned media, MODE-K cells were rinsed with Mg^{+} Ca^{++} free PBS and then incubated with HEPES and L-glutamine DMEM media (Invitrogen) containing either *Salmonella Dublin* (SD) DNA (50 μg/ml), *Bifidobacterium breve* (BB) DNA (50μg/ml), or LPS (1 μg/ml) for 1 hr. Monolayers were rinsed with Mg^{+} Ca^{++} free PBS and replaced with 5ml fresh warm high glucose with HEPES and L-glutamine DMEM media for 6 hrs. Media was removed from the basolateral compartment and used in subsequent experiments to stimulate BM-APCs.

4-2-4 APC Co- culture experiments

MODE-K cells were plated on the apical surface of Transwell supports (Costar) coated with mouse type IV Collagen (Sigma) at a concentration of $7x10^5$ cells per transwell and cultured for 2-4 days until complete monolayers were formed. MODE-K monolayers were then either co-cultured with BM-APCs ($1x10^6$ cells/ml) in the basolateral compartment underlying the monolayers [16] or exposed to various combinations of dinitrophenol (DNP: 0.1 mM), TNFα (10ng/ml; R&D Systems, Minneapolis, MN), IFNγ (10ng/ml; R&D Systems, Minneapolis, MN), BB DNA (50 μg/ml), SD DNA (50 μg/ml), or LPS (1 μg/ml). All reagents were added to the apical side of the epithelial monolayer.

4-2-5 CD4+ T-cell Isolation

Male 129Sv/ev mice between the ages of 8-12 weeks were sacrificed and the spleen harvested under sterile conditions. The organs were homogenized

between sterile frosted glass slides and rinsed with 5 ml of iMag^{tm} buffer (CMF-PBS with BSA and 0.09% sodium azide) (BD Bioscience, Mississauga ON). The cell isolates were strained through a sterile 100μm pore strainer and centrifuged at 200g for 5 min. Cell pellets were resuspended in iMag^{tm} buffer and red blood cells lysed. Splenocytes were counted and diluted to 20x10^{6}cells/ml in iMag^{tm} buffer in preparation of negative selection. Cells were selected using the BD CD4+ T-cell enrichment protocol as per manufacturer's instructions (BD Bioscience, Mississauga ON). Briefly, cells were incubated with biotinylated CD4$^+$ T-cell negative selection enrichment cocktail for 30 min on ice, then centrifuged and supernatant removed. Biotin coated magnetic beads were added to the cells and placed in the BD iMagnet for 5 min. Cells in the fraction not bound to the magnet were harvested as CD4+ T-cells and were ~ 90-95% pure. CD4+ cells were enumerated and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) to determine proliferation prior to culture conditions. Briefly, cells were incubated at 37C for 30 min in a 1.25uM solution of CFSE (Invitrogen) in iMag^{tm} buffer and the reaction quenched with RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, penicillin (1000 units/ml), streptomycin (1μg/ml), L-glutamine (2mM), 2 mercapto-ethanol (50uM), and non essential amino acids (10 mM) (Invitrogen). Cells were washed and enumerated for culture. T lymphocytes were cultured at 37C with 5% CO_2 at a concentration of 5x10^{5}cells/ml with a 1:1 concentration of anti-CD3 and CD28 coated dynabeads (Invitrogen, Oslo Norway). The cultures were stimulated with either Lipopolysaccharide (*E. coli* LPS: 1 μg/ml), *Salmonella*

dublin (SD) DNA (50 μg/ml), *Bifidobacterium breve* (BB) DNA (50 μg/ml) for 48 hrs and the cells and supernatants harvested for analysis.

4-2-6 CD4+ T-cell co-culture systems

BM-APCs were plated at 5 x 10^4cells/ml in media and incubated for 24 hrs with either Lipopolysaccharide (*E. coli* LPS: 1 μg/ml), *Salmonella dublin* (SD) DNA (50 μg/ml), *Bifidobacterium breve* (BB) DNA (50 μg/ml). CD4+ T lymphocytes were then added to the culture in a 1:10 ratio of T lymphocyte/BM-APC with 5 x 10^5cells/ml αCD3 and CD28 coated dynabeads (Invitrogen, Oslo Norway). Cells were incubated for 48hrs and supernatants harvested for analysis. For tri-culture systems, MODE-K cells were plated on the apical surface of Transwell supports (Costar) coated with mouse type IV Collagen (Sigma) at a concentration of 7x10^5 cells per transwell and cultured for 2-4 days until complete monolayers were formed. MODE-K monolayers were then co-cultured with BM-APCs (5x10^4 cells/ml) in the basolateral compartment underlying the monolayers and BB DNA (50 μg/ml), SD DNA (50 μg/ml), or LPS (1 μg/ml) applied to the apical surface. After 24hrs the MODE-K cells and the apical media were removed. CD4+ T lymphocytes were added to the culture in a 1:10 ratio of T lymphocyte/BM-APC with 5 x 10^5cells/ml anti-CD3 and anti-CD28 coated dynabeads (Invitrogen, Oslo Norway). Cells were incubated for 48hrs and supernatants harvested for analysis.

4-2-7 Analysis of cytokines and cell surface molecules

CD4+ T-cells were scraped from the bottom of the well and the suspension spun at 2000rpm for 5 min. The supernatant was collected and frozen at -70° for measurement of cytokine release by ELISA or Luminex. Levels of TGFβ (R&D systems), and IL-17(ebioscience) cytokines were analyzed by ELISA using commercially available antibodies and standards according to the manufacturers' protocols. IL-1β, IL-2, IL-4, IL-6, and TNFα were measured using mouse immune multiplex kits (Invitrogen) and a Luminex100A instrument. The cell pellets were split into two samples for measurement of expression of surface markers using a FOXp3 staining kit by manufactures instructions (Ebioscience). Briefly, cells were washed twice in PBS and stained for extracellular markers CD4, CD25 (ebioscience) for 30 min on ice then fixed in 1X fix/perm for 30 min on ice. Cells were permeabilized using 1x permeabilization buffer (eBioscience) and stained for intracellular markers FOXp3 (eBioscience) samples were analyzed using a laser flow cytometer (FACSCanto). Data was analyzed using FCS express™ software (Denovo Software).

4-2-8 Statistical Analysis

Data were tested for normality of distribution and analyses performed using the statistical software SigmaStat (Jandel Corporation, San Rafael, CA). Differences between means were evaluated using analysis of variance, student *t*-tests, or by a nonparametric Mann-Whitney Rank Sum test where appropriate. Specific differences were tested using the Dunn's or Bonferroni post hoc analysis.

4-3 Results

4-3-1Bone-marrow antigen-presenting cell phenotype

Following isolation from murine bone-marrow, two distinct groups of cells were identified by flow cytometry. The majority of the cells (~80%) were $CD11c^+$, $CD11b^+$, with ~20% of the cells being $CD11c^-$ and $CD11b^+$. This mixed population was used in the following experiments and designated as bone-marrow derived antigen-presenting cells (BM-APCs).

4-3-2 BM-APC's do not differentiate between bacterial DNA

Previous studies have shown that CpG DNA or live bacteria can induce dendritic cells to undergo maturation as evidenced by expression of cell surface markers and increased cytokine secretion.[17-18] To determine if dendritic cells would respond in a similar fashion to pathogenic and probiotic bacterial DNA, BM-APCs were incubated for 24hr with DNA from either *Bifidobacterium breve* (BB), a probiotic strain, or *Salmonella dublin* (SD), a pathogenic strain, and the release of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, GM-CSF, and TNFα measured. DNA from these bacterial strains were selected for study as we have previously demonstrated clear differences in epithelial cell response.[8-9] BM-APC response to LPS was used as a positive control. Following 24 hr incubation with bacterial DNA or LPS, all BM-APCs were CD11c+, CD11b+, MHC++, and CCR6+. BM-APCs responded to both pathogenic and probiotic bacterial DNA and to LPS with a significant increase in secretion of IL-6, IL-10, IL-12p70, and

TNFα (Table 4-1pg 79; Figure 4-1A pg 81). However, under all conditions, the secretion of IL-10 exceeded the secretion of IL-12p70 (Figure 4-1A pg 81) resulting in similar IL-10/IL-12 ratios of 2.6 ± 1.4 (Media), 5.9 ± 2.2 (BB DNA), 5.7 ± 2.1 (SD DNA), and 3.1 ± 1.3 (LPS).

n=3 replicates for all groups

Group	Condition	Cytokine					
		IL-1β (pg/ml)	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	GM-CSF (pg/ml)	TNFα (pg/ml)
BM-APCs							
	Media	22.5 ± 8.3	84.3 ± 9.2	6.5 ± 1.6	21.6 ± 5.1	31.8 ± 5.8	28.2 ± 8.5
	BB DNA	42.6 ± 5.3	85.6 ± 10.2	14.3 ± 0.7	6187 ± 868 [a]	38.0 ± 2.4	609.8 ± 52.4 [a]
	SD DNA	68.6 ± 14.4	106.4 ± 11.4	14.4 ± 2.6	7768 ± 1159 [a]	43.3 ± 2.5	824.7 ± 183.6 [a]
	LPS	60.9 ± 18.9	73.4 ± 15.2	6.5 ± 1.6	8069 ± 1132 [a]	40.4 ± 3.1	1156.2 ± 331.9 [a]
BM-APCs + MODE-K	Media	14.5 ± 0.8	51.3 ± 5.8	5.5 ± 1.0	20.25 ± 5.3	24.1 ± 1.4	7.0 ± 1.6
	BB DNA	19.6 ± 4.8	49.6 ± 10.0	7.5 ± 1.0	73.5 ± 27.7	27.1 ± 1.4	8.5 ± 2.2
	SD DNA	52.8 ± 16.6 [b]	118.5 ± 23.4 [b]	17.8 ± 6.3 [b]	6090 ± 652 [bc]	51.0 ± 4.5 [bc]	796.6 ± 277.5 [bc]
	LPS	68.2 ± 12.3 [b]	70.2 ± 4.3	8.8 ± 1.1	2934 ± 572 [bc]	44.4 ± 4.9	232.4 ± 56.2 [bc]

[a] $p<0.05$ as compared to BM-APCs - media alone
[b] $p<0.05$ compared with BM-APCs+MODE-K – media alone
[c] $p<0.05$ compared with BM-APCs+MODE-K + BB DNA

Table 4-1. Cytokine release from bone-marrow derived antigen presenting cells (BM-APCs) in the presence and absence of MODE-K cells

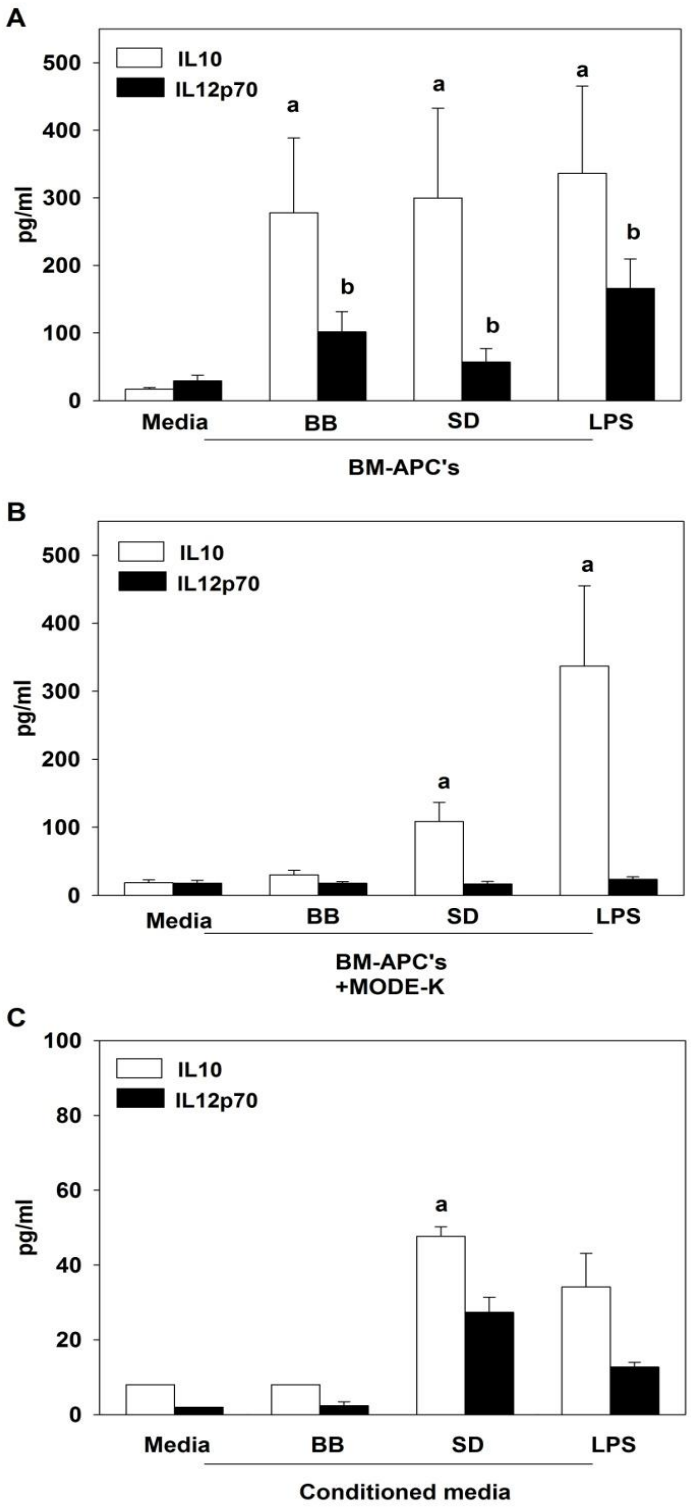

A
IL10
IL12p70
pg/ml
500
400
300
200
100
0
a
b
a
b
a
b
Media
BB
SD
LPS
BM-APC's
B
IL10
IL12p70
pg/ml
500
400
300
200
100
0
a
a
Media
BB
SD
LPS
BM-APC's
+MODE-K
C
IL10
IL12p70
pg/ml
100
80
60
40
20
0
a
Media
BB
SD
LPS
Conditioned media

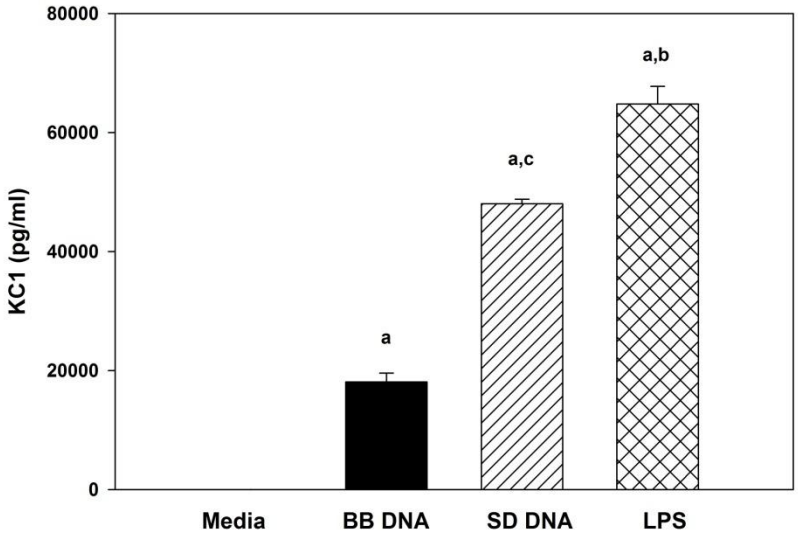
Figure 4-1

a: p<0.05 compared with media alone;
b: p<0.05 compared with control-co-culture;
c: p<0.05 compared with control-conditioned media

Figure 4-1. IL-10 and IL-12p70 secretion from BM-APCs. IL-10 and IL-12p70 secretion from BM-APCs in response to Lipopolysaccharide (LPS), *Bifidobacterium breve* (BB) DNA, and *Salmonella dublin* (SD) DNA in the absence of epithelial cells (A), in co-culture with MODE-K cells (B), or in the presence of conditioned media (CM) from MODE-K cells (C). In the absence of MODE-K cells, BM-APCs responded to BB DNA, SD DNA, and LPS with enhanced secretion of both IL-10 and IL-12p70. In the presence of MODE-K cells, secretion of IL-12p70 was suppressed in all three groups. Increased IL-10 secretion was seen in response to SD DNA and LPS, but not in response to BB DNA. Conditioned media from MODE-K cells partially suppressed IL-12p70.
n=6 for BM-APCs; n=6 for BM-APCs and MODE-K cells; n=3 for BM-APCs and conditioned media

4-3-3 MODE-K cells differentiate between bacterial DNA

To confirm that mouse intestinal epithelial cells were able to differentiate between bacterial DNA in the same manner as human epithelial cells [8-9], MODE-K cells were incubated with DNA from *S. dublin* or *B. breve* and KC1 secretion measured by ELISA. KC1 is the mouse homolog to human IL-8, and functions mainly to recruit neutrophils to sites of inflammation [19]. LPS was used as a positive control. Similar to the response seen in human HT-29 cells [9], MODE-K cells released significantly higher amounts of KC1 in response to SD DNA as compared with BB DNA (Figure 4-2 pg 83). LPS stimulation also resulted in KC1 secretion from MODE-K cells (Figure 4-2 pg 83).



a: p<0.01 compared with control;
b: p<0.01 compared with BB DNA;
c: p<0.01 compared with SD DNA

Figure 4-2. KC1 secretion from MODE-K cells. MODE-K cells were treated for 24 hrs with either LPS (1µg/ml), BB DNA (50 µg/ml) or SD DNA (50 µg/ml) and media removed for measurement of KC1 by ELISA. MODE-K cells secreted KC1 in response to all three treatments. Increased levels were secreted in response to LPS and SD DNA as compared with BB DNA.

n=4 for all conditions.

4-3-4 Epithelial cells modulate BM-APC cytokine secretion in response to apical stimulation

In the gut, bacterial DNA would normally interact with an intact epithelial cell layer prior to encountering either dendritic cells or macrophages. Thus, this next set of experiments was performed to examine whether apical stimulation of MODE-K cells with bacterial DNA would alter underlying antigen-presenting cell responses. MODE-K cells were co-cultured with BM-APCs in the underlying basolateral compartment in a transwell culture system and stimulated apically with BB DNA, SD DNA, or LPS. Cytokine secretion from BM-APCs in the basolateral compartment was measured. In this system, BM-APCs are able to sample apical contents by the extension of dendrites through pores in the transwells [2]; however, their response to bacterial DNA and LPS will be modulated by the presence of epithelial cells. The presence of MODE-K cells alone did not significantly alter cytokine secretion from BM-APCs (Table 4-1 pg 79). However, the presence of pathogenic bacterial DNA from *S. dublin* in the apical compartment overlying MODE-K cells resulted in enhanced secretion of IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, and TNFα from BM-APCs in the underlying basolateral compartment (Table 4-1 pg 79); levels which exceeded those seen when BM-APCs were directly stimulated with SD DNA. This suggests that the MODE-K cells may release a signal which augments the dendritic cell response to particular invasive pathogens. In contrast, the presence of BB DNA in the apical compartment did not elicit any response from underlying dendritic cells, even though direct contact of dendritic cells with BB DNA did induce enhanced cytokine secretion (Table 4-1 pg 79). This would

suggest that when bacteria are confined to the gut lumen, stimulation of TLR9 signaling pathways in epithelial cells will alert underlying antigen-presenting cells to the presence or absence of beneficial or pathogenic organisms in order to coordinate an appropriate immune response. These results also indicate that epithelial cells can actively suppress cytokine release from underlying antigen-presenting cells in the presence of bacterial DNA. In contrast to the differential effect of bacterial DNA on IL-1β, IL-6, IL-10 and TNFα secretion, IL-12p70 secretion from BM-APCs was suppressed by the presence of MODE-K cells, irregardless of the apical stimuli (Figure 4-1B pg 81). This is in agreement with other studies demonstrating an epithelial cell suppression of IL-12p70 secretion (17). IL-10 is considered an anti-inflammatory cytokine, where IL12p70 is a marker of inflammation, the ratio of the two cytokines can act as a marker of change from an anti-inflammatory to an inflammatory profile. This suppression of IL-12p70 secretion resulted in an enhanced ratio of IL-10 to IL-12p70 in both the SD DNA (17.6 ± 8.6) and LPS (15.9 ± 6.6) stimulated groups as compared with BB DNA stimulation (6.0 ± 5.0) and media alone (4.6 ± 2.1). LPS stimulation also resulted in a similar high release of IL-1β, IL-6, IL-10, and TNFα from dendritic cells, indicating that TLR4 signaling through epithelial cells can also modulate antigen-presenting cell function.

4-3-5 Epithelial cells modulate BM-APC function through a secreted factor

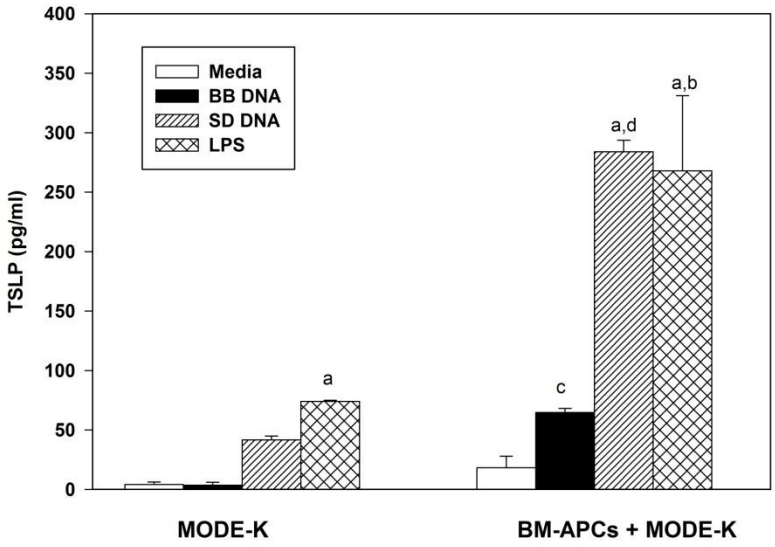
To determine if a secreted factor was released from MODE-K cells following exposure to bacterial DNA which would modulate either IL-10 or IL-12p70 secretion from underlying BM-APCs, conditioned media (CM) from DNA

and LPS-stimulated MODE-K cells was incubated with BM-APCs and IL-10 and IL-12p70 secretion measured. Under these experimental conditions, BM-APCs were not directly exposed to DNA or LPS, and any cytokine secretion that occurred would be due to signals released from MODE-K cells. Conditioned media from MODE-K cells stimulated with BB DNA did not alter either IL-10 or IL-12p70 secretion from BM-APCs (Figure 4-1C pg 81). In contrast, conditioned media from MODE-K cells that had been treated with either SD DNA or LPS stimulated both IL-10 and IL-12p70 secretion from BM-APCs (Figure 4-1C pg 81). These findings support the idea that even in the absence of direct contact; soluble mediators are released from epithelial cells in response to TLR9 signaling by pathogenic microbes that activate underlying antigen-presenting cells.

4-3-6 Role of thymic stromal lymphopoeitin

Thymic stromal lymphopoeitin (TSLP) is a cytokine secreted from epithelial cells that has been shown to be involved in the modulation of dendritic cell function and activation of T cell activity [20]. Previous studies have shown that TSLP can suppress the secretion of IL-12p70 in dendritic cells, thus switching a subsequent Th1-cell response to a Th2 type [20]. To determine if TSLP was differentially secreted in response to probiotic and pathogenic DNA, TSLP levels were measured in epithelial and co-culture supernatants. As seen in Figure 4-3 (pg 88), LPS and SD DNA stimulation of MODE-K cells resulted in the secretion of TSLP while the levels of TSLP in response to BB DNA were below the limits of the assay. Interestingly, when BM-APCs were underlying MODE-K cells, TSLP secretion was significantly enhanced in response to LPS and both

BB and SD DNA, suggesting that BM-APCs may be stimulating epithelial secretion of TSLP (Figure 4-3 pg 88). Isolated BM-APC's did not produce any detectable TSLP (data not shown). To determine if inhibition of TSLP would attenuate the MODE-K induced reduction of IL-10 and IL-12p70 release from BM-APCs, antibodies to TSLP were added to the basolateral compartment concomitantly with apical stimulation of MODE-K cells with LPS and DNA. Inhibition of TSLP removed the suppressive effect of MODE-K cells on both IL-10 and IL-12p70 secretion in response to BB and SD DNA (Table 4-2 pg 89), indicating a role for TSLP in the epithelial response to bacterial DNA.



a: p<0.01 compared with control and BB DNA treatments
b: p=0.002 compared with MODE-K LPS
c: p<0.001 compared with MODE-K BB DNA
d: p<0.05 compared with MODE-K SD DNA

Figure 4-3. TSLP secretion from MODE-K cells in the presence and absence of BM-APCs. MODE-K cells were treated for 24 hrs with either LPS (1µg/ml), BB DNA (50 µg/ml) or SD DNA (50 µg/ml) and media removed for measurement of TSLP by ELISA. MODE-K cells secreted barely detectable levels of TSLP in the absence of microbial stimuli. TSLP secretion was enhanced in the presence of BM-APC's

Condition	Stimulus	IL10 (%)	IL12p70 (%)
BM-APCs + MODE-K			
	Media	100	100
	BB DNA	170 ± 38	113 ± 28
	SD DNA	521 ± 157	96 ± 3
	LPS	2439 ± 1840	161 ± 43
BM-APCs + MODE-K + ANTI-TSLP	Media	100	100
	BB DNA	1215 ± 182	1292 ± 483
	SD DNA	1288 ± 381	3121 ± 771[a]
	LPS	1258 ± 738	2054 ± 538

[a] p<0.02 as compared to media alone

Table 4-2. Cytokine release from bone-marrow BM-APCs in co-culture with MODE-K cells when stimulated with LPS and bacterial DNA in the presence and absence of anti-TSLP n=3 for all groups

4-3-7 Pathogenic bacterial DNA induces T effector cell differentiation

To examine whether bacterial DNA stimulation of epithelial cells would be translated into a BM-APC-mediated altered CD4+ T cell differentiation, a tri-culture system was used whereby MODE-K cells were incubated with apical bacterial DNA and then with BM-APCs and CD4+ T cells. In the absence of MODE-K cells, BM-APCs and CD4+ T cells incubated with SD DNA and BB DNA responded with enhanced IL-10 (Figure 4-4A pg 92) and IL-17 secretion (Figure 4-4B pg 92), indicative of a reduced Th1 and enhanced Th17 response. IFNγ secretion was not affected by either SD or BB DNA (Figure 4-4C pg 92). However, in the presence of epithelial cells, SD DNA stimulation of MODE-K cells resulted in an increase in both IL-17 and IFNγ secretion from CD4+ T cells. In contrast, BB DNA treatment of MODE-K cells did not alter either IL-17 or IFNγ secretion from T cells.

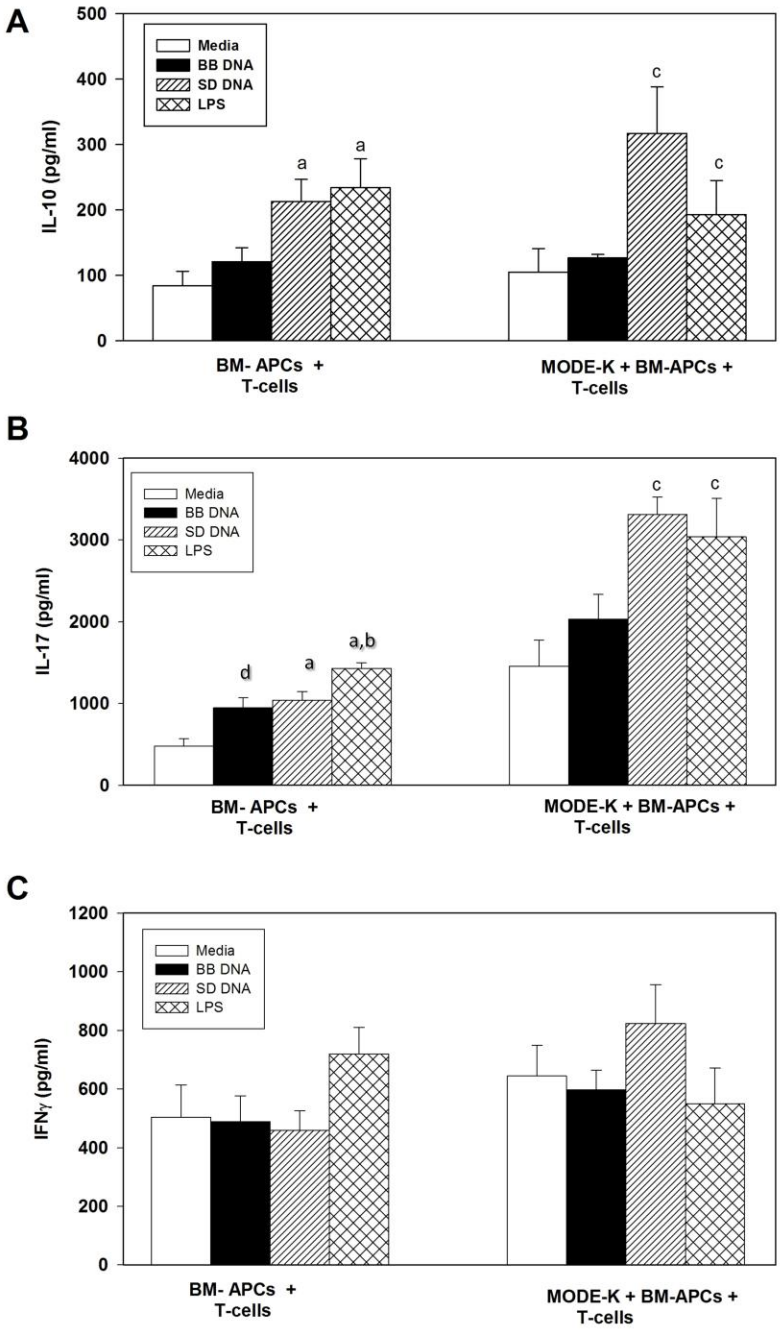

A
IL-10 (pg/ml)
500
400
300
200
100
0
Media
BB DNA
SD DNA
LPS
a
a
c
c
BM- APCs +
T-cells
MODE-K + BM-APCs +
T-cells
B
IL-17 (pg/ml)
4000
3000
2000
1000
0
Media
BB DNA
SD DNA
LPS
d
a
a,b
c
c
BM- APCs +
T-cells
MODE-K + BM-APCs +
T-cells
C
IFNγ (pg/ml)
1200
1000
800
600
400
200
0
Media
BB DNA
SD DNA
LPS
BM- APCs +
T-cells
MODE-K + BM-APCs +
T-cells

Figure 4-4

a: p<0.01 compared with control and BB DNA treatments
b: p=0.025 compared to SD treated BM-APC's+ T-cells
c: p<0.001 compared to BB Treated MODE-K+ BM-APC's +T-cells
d: p<0.001 compared to Control BM-APC's + T-cells

Figure 4-4 Cytokine secretion from CD4+ Cells. IL-10(A), IL-17(B), IFNg (C) secretion from CD4+ cells Incubated with BM-APC's treated with LPS (1ug/ml) BB DNA(50ug/ml), SD DNA(50ug/ml). IL-17 secretion was increased in response to all three stimuli. In the prescence of MODE-K cells SD DNA and LPS enhanced IL-17 secretion while BB DNA had no effect. IFNg secretion was increased when BM-APC's were stimulated with LPS. In the prescence of MODE-K cells, IFNg secretion was decreased in the prescence of LPS and enhanced in the prescence of SD DNA, But not in the prescence of BB DNA.

4- 4 Discussion

In this study, we demonstrate that murine intestinal epithelial cells respond in a differentially fashion to probiotic and pathogenic bacteria derived DNA and this differentiation is communicated to underlying bone-marrow antigen presenting cells resulting in altered cytokine secretion. Further, apical stimulation of epithelial cells with pathogenic bacterial DNA, but not probiotic bacterial DNA, results in the enhancement of IL-17 and IFNγ secretion from underlying T cells, suggesting an induction of effector T cell subsets. In that bone-marrow derived antigen presenting cells, in the absence of epithelial cells, do not discriminate between bacterial DNA species, this highlights the important role that epithelial cells have in modulating innate and adaptive immune responses to gut microbes.

In the gut, epithelial cells are constantly exposed to high numbers of both commensal and pathogenic microbes. In epithelial cells, the primary means of recognition of luminal microbes is through innate pattern recognition receptors, including Toll like receptors (TLRs) and nucleotide-binding oligomerization domains (Nods).[5] TLR9 is expressed on the cell surface of epithelial cells,[7] and recognizes unmethylated 2' deoxyribocytosine-phosphate-guanine (CpG) motifs found in high amounts in bacterial DNA.[4] Oral administration of probiotic bacteria has shown benefit in various conditions, including some forms of inflammatory bowel disease and atopic diseases.[21] Some studies have shown TLR9 signaling to be required for the anti-inflammatory effects of probiotics,[11] and TLR9 signaling has also been associated with maintenance of gut homeostasis.[7] The finding that different bacterial strains can modulate dendritic

cell and T cell differentiation through their DNA and interactions with epithelial cells provides a mechanism to help explain these findings.

It is clear that epithelial and immune cells respond in a differential fashion to bacterial DNA. In this study, we have shown that epithelial cells responded to pathogenic bacterial DNA with inhanced KC1 and TSLP secretion, while probiotic bacterial DNA did not elicit any TSLP secretion, and a minimal amount of KC1. In contrast, antigen-presenting cells isolated from murine bone marrow responded to both probiotic and pathogenic bacterial DNA with increased IL-6, IL-10, IL-12p70, and TNFα secretion, suggesting a strong Th1 skewing milieu. These findings are in agreement with those of Hall et al,[12] who showed gut flora DNA to induce IL-6 production in isolated dendritic cells. These different responses by epithelial cells and antigen-presenting cells to bacterial DNA may be due to the cellular location of TLR9. In dendritic cells, TLR9 is expressed in intracellular compartments, and internalization and endosomal acidification is necessary for DNA to activate TLR9 signaling.[4] In contrast, we, and others, have shown TLR9 to be expressed on the surface of epithelial cells, and endosomal acidification is not required for TLR9 activation,[7-8] suggesting the existence of an alternate TLR9 signaling pathway in epithelial cells that can differentiate bacterial DNA based on pathogenicity.

Antigen-presenting cells, in particular, dendritic cells and macrophages, are key gate keeping cells of the immune system and act as sentinels in the intestinal tract. Dendritic cells have the unique ability to induce primary immune responses against pathogenic microbial invasion and also to maintain

immunological tolerance. Dendritic cells modulate adaptive T and B cell responses by recognizing pathogens either directly or indirectly through sensing perturbations in the mucosal environment. Under normal conditions, sub epithelial dendritic cells in the gut continually sample the contents of the gut lumen by passing their dendrites between epithelial cells[22] and also by interacting directly with bacteria that have gained access to either the lamina propria or Peyer's patches.[23] Depending upon the stimulus, dendritic cells stimulate naïve T cells towards either T helper type 1 (Th1), Th2, Th17, or T regulatory cell phenotypes. Hall and colleagues[12] have recently shown that dendritic cells stimulated with gut flora derived DNA suppressed conversion of Treg cells and increased both Th1 and Th17 cells. Our data supports and extends these findings, in that antigen-presenting cells stimulated with DNA from *Salmonella dublin* enhanced both IL-17 and IFNγ secretion from CD4+ T cells. In addition, our results clearly demonstrate that DNA from different bacterial strains differs in their capacity to elicit responses through TLR9 signaling when epithelial cells are present. These findings have important implications for how the gut maintains homeostasis under normal conditions. Under normal conditions, microbes are contained within the lumen and interact with dendritic cells in the presence of epithelial cells. However, under inflammatory conditions where there is a breach in the gut barrier, an influx of luminal microbes would interact with dendritic cells in the absence of epithelial cell signals, and a substantially different response may occur. Further, patients with inflammatory bowel disease have altered gut microflora,[24] thus the presence of more inflammatory

bacterial species may drive the differentiation of Th17 cells in these patients.[25-26]

In the gut, interactions between epithelial cells and underlying antigen-presenting cells occur via direct contact and also through the release of chemokines/cytokines. In agreement with previous studies,[20] we found that MODE-K cells effectively suppressed IL-12p70 secretion, and further, thymic stromal lymphopoeitin (TSLP) played a role in this suppression. Both *B. breve* DNA and *S. dublin* DNA induced the secretion of TSLP from MODE-K cells, and inhibiting the actions of TSLP with an antibody resulted in a large increase in IL-12p70 secretion from BM-APCs. Interestingly, neutralization of TSLP also resulted in a significant increase in IL-10 secretion as well, indicating a role for TSLP in modulating IL-10 secretion as well.

A role for soluble mediators in modulating BM-APC cytokine secretion was seen in the response of BM-APCs to conditioned media. In these experiments, MODE-K cells were treated with DNA, and conditioned media was removed from the basolateral compartment and added to BM-APCs. Even in the absence of any direct contact of BM-APCs with bacterial DNA, conditioned media from MODE-K cells that had been stimulated with *S. dublin* DNA, but not *B. breve* DNA, stimulated both IL-10 and IL-12p70 secretion from BM-APCs.

In conclusion, the ability of intestinal epithelial cells to distinguish between harmless commensal and pathogenic microbes solely by their DNA and transmit this information to underlying immune cells is critical for homeostasis and host

defense. Here we present evidence that bacterial DNA can dictate the manner in which the intestinal immune system and epithelial cells respond to different bacterial species. Indeed, we demonstrate that intestinal epithelial cells may have evolved a strategy to distinguish between pathogenic, commensal, and probiotic bacteria based upon a bacterial DNA fingerprint, and furthermore, may pass this information to underlying dendritic cells and T cells. Furthermore, we also describe a potentially important mechanism by which probiotic bacteria may elicit immunomodulatory effects.

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Chapter 5 Bacteria DNA modulates immune activity under inflammatory conditions *in vitro* but not *in vivo*

5-1 Introduction

Inflammatory bowel diseases (IBD) are diseases of unknown etiology that involve dysregulated immune responses to commensal microflora. Current treatments aim to reduce symptoms and inflammation. The most common treatments for IBD include immunosuppressive drugs, anti-inflammatory medications, and biological therapy. These treatments are costly and have various negative side effects.[1-2] Thus the search continues for cost effective safe treatments.

Probiotics are “living micro-organisms which, when consumed in adequate quantities, confer a health benefit on the host”.[3] Probiotics have been used to treat IBD with limited success; this may be due to the use of inappropriate probiotic strains in trials as well as the differences in clinical trial size, treatment regimes, and protocols.[4-5] In contrast to the limited success of probiotic therapy in human IBD trials, a large body of evidence using *in vitro* and animal models of IBD have shown that *Bifidobacteria* probiotics have anti-inflammatory properties, tighten the epithelial barrier, and are able to both prevent, and treat, colitis.[6] There is evidence that probiotics impart their beneficial effects through numerous mechanisms, including secreted bioactive factors[7], modulation of intracellular

signaling pathways, competitive inhibition, and by DNA signaling through TLR9 receptors[8].

Intestinal epithelial cells (IEC's) experience the outside world through innate immune receptors including TLR's. Surface TLR9 recognizes un-methylated CpG motifs found in a high proportion of bacterial DNA.[9] It has been shown that IEC's release soluble factors that condition underlying dendritic cells to prime $CD4^+$ T helper cells to undergo type-2 (Th2) differentiation and secrete IL-4 and IL-10 in response to live and irradiated bacteria.[10-11] Rimoldi et al[10] have shown that gut dendritic cells are conditioned by factors derived from epithelial cells to become "noninflammatory". The dendritic cells are unable to release IL-12 and activate inflammatory Th1 cells highlighting the role of IEC's in maintaining homeostasis.

In the previous chapter I demonstrated that probiotic bacterial DNA induces an altered cytokine profile from antigen-presenting cells (APC's) through an IEC-mediated process. Further, we demonstrated a reduction in downstream secretion of IL-17a in an *in vitro* tri-culture system, suggesting that probiotic bacterial DNA may initiate its anti-inflammatory functions by affecting the Th17 inflammatory cascade. However, whether bacterial DNA would have the same effects under inflammatory conditions is unknown. Previous studies have demonstrated that apical vs. basolateral signaling by TLR9 results in a differential response by IECs.[12] Thus, under conditions of increased permeability and active inflammation, interactions of bacterial DNA with the basolateral surface may result in a different response. Further, it has been shown that under

conditions of metabolic stress that would exist under acute inflammatory conditions, IECs respond in a different fashion to normal commensal bacteria.[13] In these next series of experiments, we explored the effect of pro-inflammatory cytokines and metabolic stress on the interactions between IEC's, bacterial DNA, and downstream APC immune function in a tri-culture model system. Further, we carried out a series of *in vivo* experiments by treating 129 Sv/Ev wild-type and IL-$10^{-/-}$mice with bacterial DNA

5-2 Materials and Methods

5-2-1 Bacterial strains and preparation of DNA

Salmonella enterica serovar dublin strain Lane (ATCC #15480) was selected for these studies as a pathogenic bacteria due to the ability of its DNA to induce IL-8 secretion from intestinal epithelial cells.[14] *Bifidobacteria breve* Y8 (VSL Pharmaceutics) was selected as a probiotic bacteria due to the ability of its DNA to reduce basal IL-8 secretion.[14] Bacteria were inoculated at 0.18% (v/v) into 25 ml of Mann-Rogosa Sharpe broth (Difco #0370-17-3) and grown statically overnight (18 – 20 h) at 37°C. For DNA isolation, cells were centrifuged at 11700*g* for 10 min, washed with SSC buffer (0.15 NaCl, 0.015 M sodium citrate) and re-suspended in 0.01M sodium phosphate buffer with 20% sucrose and 2.5 mg/ml lysozyme for 45 min at 37°C followed by lysis buffer (10mM Tris-HCl, 1mM EDTA, 500 mg pronase B, 1% SDS, pH 8) for 30 min at 37°C. DNA was extracted by adding an equal volume of 1:1 buffer-saturated phenol and chloroform to the bacterial solution. The mixture was spun for 5 min at 4000 rpm,

and the aqueous layer removed. The extraction was repeated until no interface was visible. Traces of phenol were removed with chloroform and the salt concentration adjusted by the addition of 1/10 volume of sodium acetate, pH 5.2. DNA was precipitated with cold 100% ethanol, washed with 70% ethanol and re-suspended in sterile Tris-EDTA buffer. A post isolation treatment to remove endotoxin was performed with a 5% volume of Tx114 (Promega) at 4ºC for 30 min. Further isopropanol precipitation was done to isolate endotoxin-free DNA. Only preparations with endotoxin levels not exceeding 0.1ng endotoxin per µg DNA were used. Concentration and purity of DNA preparations was confirmed by measuring OD_{260} absorbance, $OD_{260/280}$ ratio, and by running agarose gel electrophoresis as previously described [14]. DNA preparations were assayed for endotoxin using the *Pyrochrom* amebocyte assay (DP0704, Cape Cod Inc, MA, and USA). DNase treated preparations were used in all experiments as control preparations. DNA preparations were incubated overnight at 37°C with 5 mg/ml DNase 1 (Sigma-Aldrich) in the presence of 5mM $MgCl_2$. DNA depletion was confirmed by agarose gel electrophoresis with ethidium bromide staining as previously shown [8]. Prior to cell culture treatment, DNase was heat-inactivated at 90ºC for 10 min.

5-2-2 Bone Marrow- Derived Antigen Presenting Cell Isolation

APC's cells were isolated from the long bones of male 129Sv/ev mice using a modified protocol [15]. Briefly, long bones of mice were isolated and cleaned, then placed in 70% ETOH for 5 min and transferred to calcium and magnesium-free PBS (CMF-PBS). The ends of bones were clipped and the

marrow rinsed with 10ml CMF-PBS. Cells were spun @ 2000 RPM for 5 min, re-suspended in CMF-PBS and plated at 2 x 10^6 in 10 ml media. Cells were maintained at 37° C with 5% CO_2 in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, penicillin (1000 units/ml), streptomycin (1μg/ml), L-glutamine (2mM) (Invitrogen) and 1000U/ml mouse recombinant GM-CSF (R&D). After 10 days of incubation, the cellular mixture was stained with $CD11c^+$, $CD11b^+$ and 7ADD to determine viability. Following, incubation, two primary groups of cells were identified by flow cytometry. The majority of the cells (~80%) were CD11b+ and CD11c+, while ~20% of cells were CD11b+ and CD11c- (Figure 2-1 pg 36) and were thus defined as bone-marrow derived antigen presenting cells (BM-APCs). On day 10, the cells were counted and plated at a concentration of 1x10^6 cells/ml without mrGM-CSF. BM-APC's were then incubated for 24 hrs with either Lipopolysaccharide (*E. coli* LPS: 1 μg/ml), *Salmonella dublin* (SD) DNA (50 μg/ml), *Bifidobacterium breve* (BB) DNA (50 μg/ml), or conditioned media derived from MODE-K cells (CM). Following incubation, flow cytometry analysis demonstrated that all cells were positive for CD11c, CD11b, CCR6, and CD8α and showed up-regulation of surface MHCII IA/IE marker (Figure 2-1 pg 36).

5-2-3 MODE-K Mouse Small Intestinal Cells

Mouse intestinal cells (MODE-K) immortalized with mouse SV40 large T gene transfer with mouse ectopic virus exhibit similar morphological and phenotypic characteristics of normal enterocytes [16] and were used for these studies. The cells were a generous gift from Dr. K Croitoru (University of Toronto,

Toronto, ON). The cells were maintained at 37º C and 5% CO_2 in DMEM high glucose with HEPES and L-glutamine (Invitrogen) supplemented with 10% heat inactivated FBS. To prepare conditioned media, MODE-K cells were rinsed with Mg^{+} Ca^{++} free PBS and then incubated with HEPES and L-glutamine DMEM media (Invitrogen) containing either *Salmonella Dublin* (SD) DNA (50 µg/ml), *Bifidobacterium breve* (BB) DNA (50µg/ml), or LPS (1 µg/ml) for 1 hr. Monolayers were rinsed with Mg^{+} Ca^{++} free PBS and replaced with 5ml fresh warm high glucose with HEPES and L-glutamine DMEM media for 6 hrs. Media was removed from the basolateral compartment and used in subsequent experiments to stimulate BM-APCs.

5-2-4 APC Co- culture experiments

MODE-K cells were plated on the apical surface of Transwell supports (Costar) coated with mouse type IV Collagen (Sigma) at a concentration of $7x10^5$ cells per transwell and cultured for 2-4 days until complete monolayers were formed. MODE-K monolayers were then either co-cultured with BM-APCs ($1x10^6$ cells/ml) in the basolateral compartment underlying the monolayers [17] or exposed to various combinations of dinitrophenol (DNP: 0.1 mM), TNFα (10ng/ml; R&D Systems, Minneapolis, MN), IFNγ (10ng/ml; R&D Systems, Minneapolis, MN), BB DNA (50 μg/ml), SD DNA (50 μg/ml), or LPS (1 μg/ml). All reagents were added to the apical side of the epithelial monolayer.

5-2-5 CD4+ T-cell Isolation

Male 129Sv/ev mice between the ages of 8-12 weeks were sacrificed and the spleen harvested under sterile conditions. The organs were homogenized between sterile frosted glass slides and rinsed with 5 ml of iMag^{tm} buffer (CMF-PBS with BSA and 0.09% sodium azide) (BD Bioscience, Mississauga ON). The cell isolates were strained through a sterile 100µm pore strainer and centrifuged at 200g for 5 min. Cell pellets were resuspended in iMag^{tm} buffer and red blood cells lysed. Splenocytes were counted and diluted to 20x10^{6}cells/ml in iMag^{tm} buffer in preparation of negative selection. Cells were selected using the BD CD4+ T-cell enrichment protocol as per manufacturer's instructions (BD Bioscience, Mississauga ON). Briefly, cells were incubated with biotinylated CD4^{+} T-cell negative selection enrichment cocktail for 30 min on ice, then centrifuged and supernatant removed. Biotin coated magnetic beads were added to the cells and placed in the BD iMagnet for 5 min. Cells in the fraction not bound to the magnet were harvested as CD4+ T-cells and were ~ 90-95% pure. CD4+ cells were enumerated and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) to determine proliferation prior to culture conditions. Briefly, cells were incubated at 37C for 30 min in a 1.25uM solution of CFSE (Invitrogen) in iMag^{tm} buffer and the reaction quenched with RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, penicillin (1000 units/ml), streptomycin (1µg/ml), L-glutamine (2mM), 2 mercapto-ethanol (50uM), and non essential amino acids (10 mM) (Invitrogen). Cells were washed and enumerated for culture. T lymphocytes were cultured at 37C with

5% CO_2 at a concentration of $5x10^5$cells/ml with a 1:1 concentration of anti-CD3 and CD28 coated dynabeads (Invitrogen, Oslo Norway). The cultures were stimulated with either Lipopolysaccharide (*E. coli* LPS: 1 μg/ml), *Salmonella dublin* (SD) DNA (50 μg/ml), *Bifidobacterium breve* (BB) DNA (50 μg/ml) for 48 hrs and the cells and supernatants harvested for analysis.

5-2-6 Animals and Treatments

Male 129Sv/ev and 129Sv/ev IL-10-/- mice aged 8-12 weeks of age were fed daily with *Salmonella dublin* (SD) DNA (50 μg/ml), *Bifidobacterium breve* (BB) DNA (50 μg/ml), or vehicle (ddH_20) for 7 days. On day 8 animals were sacrificed and colon, spleen, and mesenteric lymph nodes (MLN) harvested. Colons were processed for histology, and homogenized for Luminex analysis. Spleens and MLN were homogenized between sterile frosted glass slides and rinsed with 5 ml of iMag^{tm} buffer (BD Bioscience, Mississauga ON). (CMF-PBS with BSA and 0.09% sodium azide). The cell isolates were strained through a sterile 100um pore strainer and centrifuged at 200g for 5 min. Cell pellets were resuspended in iMag^{tm} buffer. Spleen cells isolated were exposed to a red blood cell lysis and centrifuged at 200g for 5 min. Both MLN and spleen cells were enumerated and diluted to $20x10^6$cells/ml in iMag^{tm} buffer in preparation of negative selection. Cells were selected using the BD CD4+ T-cell enrichment protocol. Briefly, cells were incubated with biotinylated $CD4^+$ T-cell, negative selection enrichment cocktail for 30 min on Ice. Cells were centrifuged at 200g for 5 min and supernatant removed. Biotin coated magnetic beads were added to the cells and

incubated for 20min at 4C. The cells were brought up to 2ml in iMag buffer and placed in the BD iMagnet for 5 min. Cells in the fraction not bound to the magnet were harvested as CD4+ T-cells. The cells bound to the magnet were rinsed with 2ml iMag^{tm} and placed in the magnet for 5 min and the flow through collected and pool with the first fraction. The cells were on average 90-95% pure CD4+ T-cell cultures. CD4+ T-cell cultures were incubated for 48hrs and activated with human anti-CD3. Supernatants were collected for Luminex analysis and cells were harvested for either proliferation or flow cytometry.

5-2-7 Cellular Proliferation

At 48 hrs of culture half the supernatant was removed from each well and frozen @ -70 for analysis by ELISA or Luminex. An equal volume of warm media containing 10uCi H^3 thymidine was added to the culture and incubated for 24 hrs. Cells were harvested using an Inotech cell harvester and read on Wallac MicroBeta TriLux scintillation counter.

5-2-8 Analysis of cytokines and cell surface molecules

CD4+ T-cells were scraped from the bottom of the well and the suspension spun at 2000rpm for 5 min. The supernatant was collected and frozen at -70° for measurement of cytokine release by ELISA or Luminex. Levels of TGFβ (R&D systems), and IL-17(ebioscience) cytokines were analyzed by ELISA using commercially available antibodies and standards according to the manufacturers' protocols. IL-1β, IL-2, IL-4, IL-6, and TNFα were measured using mouse immune multiplex kits (Invitrogen) and a Luminex100A instrument. The

cell pellets were split into two samples for measurement of expression of surface markers using a FOXp3 staining kit by manufactures instructions (Ebioscience). Briefly, cells were washed twice in PBS and stained for extracellular markers CD4, CD25 (ebioscience) for 30 min on ice then fixed in 1X fix/perm for 30 min on ice. Cells were permeabilized using 1x permeabilization buffer (eBioscience) and stained for intracellular markers FOXp3 and IL-17 (eBioscience) samples were analyzed using a laser flow cytometer (FACSCanto). Data was analyzed using FCS express™ software (Denovo Software).

5-2-9 Statistical Analysis

Data were tested for normality of distribution and analyses performed using the statistical software SigmaStat (Jandel Corporation, San Rafael, CA). Differences between means were evaluated using analysis of variance, student *t*-tests, or by a nonparametric Mann-Whitney Rank Sum test where appropriate. Specific differences were tested using the Dunn's or Bonferroni post hoc analysis.

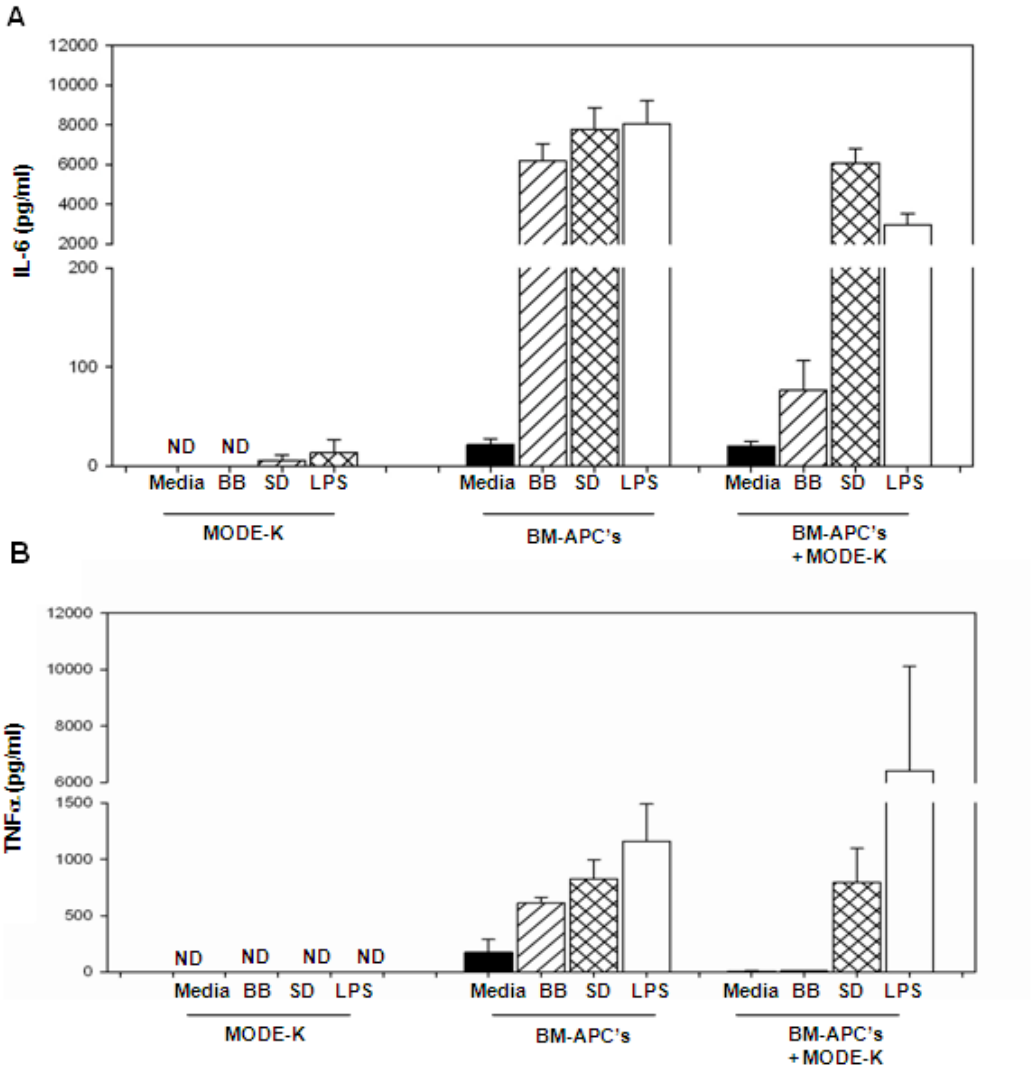
5-3 Results

5-3-1 Pro-inflammatory cytokines alter epithelial cell modulation of BM-APC cytokine release

Dysregulation of immune responses to normal commensal microbes can result in chronic intestinal inflammation, and there is evidence that dendritic cells may be directly involved in this dysregulation.[18] Previous studies have

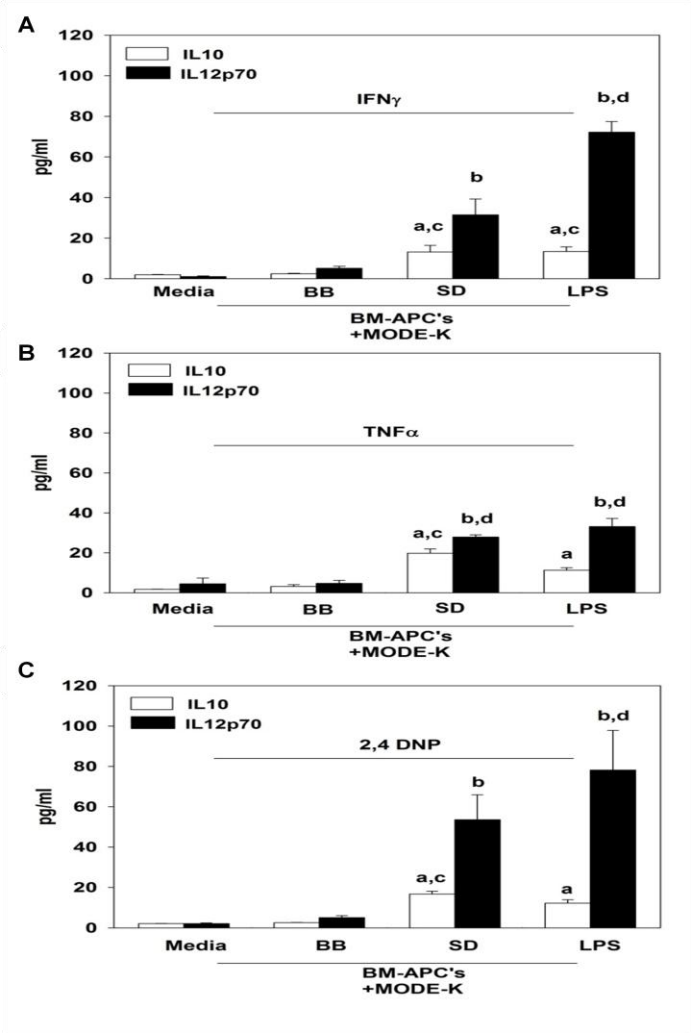
demonstrated that under metabolic stress and mild inflammation, intestinal epithelial cells respond in a differential fashion to bacteria.[13, 19] To determine if this altered epithelial response would extend to TLR9 ligands, MODE-K cells were treated with IFNγ or TNFα prior to bacterial DNA; LPS treatment was used as a positive control. Pre-treatment of MODE-K cells with IFNγ resulted in a substantial increase in IL-6, IL-10, IL-12p70, and TNFα secretion from BM-APCs following stimulation of MODE-K cells with either SD DNA or LPS (Table 5-1 pg 116). In contrast, treatment of MODE-K cells with BB DNA, while stimulating a small rise in IL-6 levels from BM-APCs, did not result in secretion of IL-10, IL-12p70, or TNFα from BM-APCs. A different profile was seen when MODE-K cells were pre-treated with TNFα (Table 5-1 pg 116). In this group, subsequent treatment with BB DNA, SD DNA, and LPS all resulted in a similar rise in IL-6 levels, while SD DNA and LPS treatment both increased IL-10, IL-12p70, and TNFα values significantly more than did BB DNA. The rise in IL-6 and TNFα was determined to be due to BM- APC's as IL-6 and TNFα were not detected in DNA and LPS stimulated MODE-K cultures (Figure 5-1 pg 112). Both TNFα and IFNγ treatment of MODE-K cells and subsequent SD DNA and LPS treatment resulted in a ~10 fold reduction in the amount of IL-10 secreted from BM-APCs under co-culture conditions and an increased IL-12p70 release (Figure 5-2A, B pg 113). Treatment of MODE-K cells with IFNγ (Figure 5-2A pg 113) or TNFα (Figure 5-2C pg 113) also altered the ratio of IL-10 to IL-12p70 secretion, with IL-12p70 levels increasing compared with IL-10 in all treatment groups. To determine if the increase in IL-12p70 could be due to a suppression of TSLP secretion from

MODE-K cells in response to TNFα or IFNγ treatment, levels of TSLP were measured in the basolateral compartment. As seen in figure 5-3 (pg 114), treatment of MODE-K cells with either TNFα or IFNγ attenuated the levels of TSLP secretion, suggesting that this decrease could have a role in the increased IL-12p70 secretion. However, no TSLP was detected in response to BB DNA. Little IL-12p70 was secreted in response to BB DNA this combined with the lower levels of TNFα secreted further demonstrating the ability of IEC's to maintain a less inflammatory profile in response to probiotic bacteria DNA under inflammatory conditions (Figure 5-1pg 112, 5-2 pg 113).



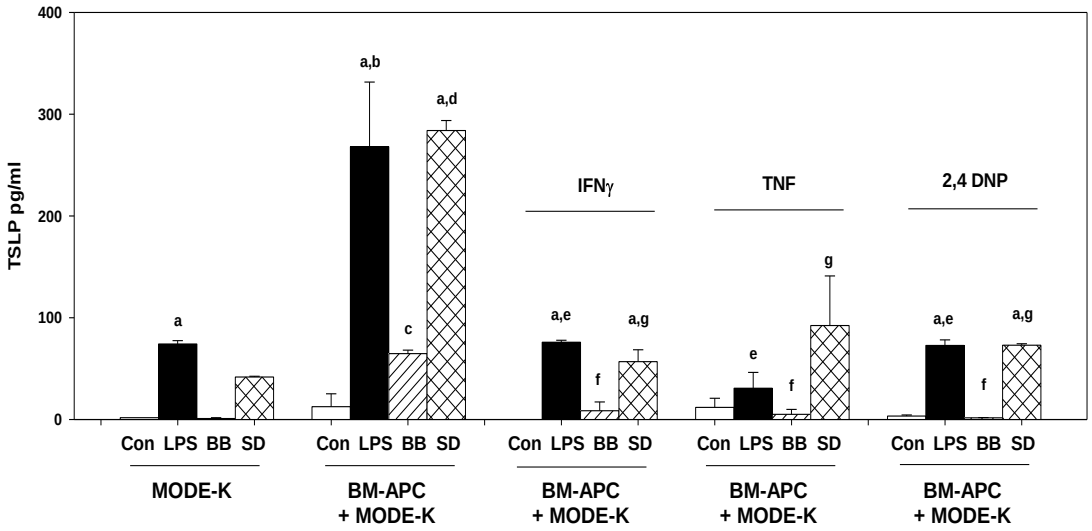
ND=not detected

Figure 5-1 Cytokine secretion from MODE-K IEC's, bone-marrow derived BM-APCs (BM-APCs) in co-culture (BM-APCs + MODE-K IL-6 (A) and TNFα (B) secretion from MODE-K, BM-APC's, and co-culture cells in response to Lipopolysaccharide (LPS), *Bifidobacterium breve* (BB) DNA, and *Salmonella dublin* (SD) DNA



a: p<0.05 as compared to IL-10 secretion Media
b: p<0.05 as compared to IL-12 secretion Media
c: p<0.05 as compared to IL-10 secretion BB DNA
d: p<0.05 as compared to IL-12 secretion BB DNA

Figure 5-2. Cytokine secretion from bone-marrow derived BM-APCs in co-culture (BM-APCs + MODE-K) in the presence of pro-inflammatory cytokines and metabolic stress. IL-10 and IL-12p70 secretion from BM-APCs-MODE-K co-culture in response to Lipopolysaccharide (LPS), *Bifidobacterium breve* (BB) DNA, and *Salmonella dublin* (SD) DNA in the presence of IFNγ (100ng/ml), TNFα (20ng/ml) or 0.1 mM 2,4 di-nitrophenol (2,4 DNP). MODE-K cells were pre-treated with cytokines or 2, 4-DNP for 1 hour prior to LPS, BB or SD DNA treatment. All three treatments resulted in a ~10 fold decrease in levels of IL-10 released from BM-APCs (Figure 4-1 pg 81) n=3 for all conditions.



a: p<0.01 compared with control and BB DNA treatments;
b: p=0.002 compared with MODE-K LPS **;**
c: p<0.001 compared with MODE-K BB DNA;
d: p<0.05 compared with MODE-K SD DNA;
e: p<0.05 compared with BM-APC+MODE-K LPS;
f: p<0.05 compared with BM-APC+MODE-K BB;
g: p<0.05 compared with BM-APC+MODE-K SD

Figure 5-3. TSLP secretion from MODE-K cells in the presence and absence of BM-APCs and inflammatory cytokines. MODE-K cells were treated for 24 hrs with LPS (1µg/ml), BB DNA or SD DNA (50 µg/ml) and media removed for measurement of TSLP by ELISA. MODE-K cells secreted barely detectable levels of TSLP in the absence of microbial stimuli. TSLP secretion was enhanced in the presence of BM-APC's

5-3-2 Metabolically stressed epithelial cells modulate BM-APC profile shift

Under conditions of acute and chronic inflammation, epithelial cells demonstrate various levels of metabolic stress due to the presence of reactive nitrogen species and pro-inflammatory cytokines resulting in a pathogenic factor for IBD.[13, 20] To determine if an underlying alteration in metabolic stress was responsible for the effects observed in the presence of IFNγ and TNFα, MODE-K cells were treated with 2,4 DNP prior to bacterial DNA and LPS challenge. Under these conditions, a very similar response to that seen in response to IFNγ was observed as SD DNA and LPS stimulation of MODE-K cells resulted in a higher IL-6, IL-10, IL-12p70, and TNFα secretion (Table 5-1 pg 116) and a lower IL-10 to IL-12 ratio (Figure 5-2C pg 113).

Pre-treatment	*Stimulus*	*Cytokine*			
		IL-4 (pg/ml)	**IL-6 (pg/ml)**	**GM-CSF (pg/ml)**	**TNFα (pg/ml)**
IFNγ	**Media**	9 ± 2	11 ± 3	ND	13 ± 0
	BB DNA	8 ± 2	534 ± 209[a]	ND	19 ± 6
	SD DNA	6 ± 1	3075 ± 783[ab]	ND	380 ± 9 [ab]
	LPS	10 ± 2	3540 ± 783[ab]	56 ± 29	377 ± 148[ab]
TNFα	**Media**	6 ± 3	61 ± 34	ND	17.3 ± 2.1
	BB DNA	8 ± 2	3151 ± 644[a]	ND	98 ± 45
	SD DNA	8 ± 2	3328 ± 488[a]	ND	260 ± 84[ab]
	LPS	7 ± 3	3103 ± 550[a]	49 ± 11	297 ± 34[ab]
2,4-DNP	**Media**	3 ± 2	8 ± 1	ND	13.8 ± 0.3
	BB DNA	7 ± 2	444 ± 183[a]	ND	15.6 ± 1.0
	SD DNA	4 ± 1	2772 ± 224[ab]	ND	200 ± 55[ab]
	LPS	9 ± 1	2347 ± 281[ab]	ND	154 ± 25[ab]

ND= not detected

a: p<0.05 as compared to media
b: p<0.05 as compared to BB DNA treatment

Table 5-1. Cytokine release from bone-marrow BM-APCs in co-culture with MODE-K cells pre treated with IFNγ, TNFα, or 2,4 DNP. Cytokines secreted into the basolateral compartment of MODE-K and BM- APC co cultures after a 1 hr pre treatment with either 10ng/ml IFNγ, TNFα, or 0.1mM 2,4DNP and subsequent activation by either 50ug DNA from BB or SD or 1ug/ml LPS determined by Luminex or ELISA

5-3-3 IL-10 deficient mice have increased colonic cytokines prior to disease.

10 week old male WT and IL-$10^{-/-}$ mice were fed daily 50ug of either pathogenic bacteria DNA (*Salmonella Dublin*) or probiotic bacteria DNA (*Bifidobacterium breve*) for seven days and colonic tissue homogenates analyzed for cytokine levels by Luminex and ELISA. IL-$10^{-/-}$ mice had significantly greater colonic levels of IL-4, IL-12p35/p40, and IL-17a compared with WT mice. In addition, WT and IL-$10^{-/-}$ mice responded differently to bacterial DNA treatment.

In WT mice treated with bacterial DNA, there was a trend towards increased levels of IL-2 and IL-12p35/40 in colonic tissue from mice fed either SD DNA or BB DNA. In contrast, WT mice fed BB DNA had a trend towards increased IL-2 and decreased TGFβ levels, while WT mice fed SD DNA had a trend towards increased IL-2 and increased TGFβ levels (Table 5-2 pg 118).

In IL-$10^{-/-}$ mice treated with BB DNA, there was a trend towards decreased IL-2, and IL-4, and increased TGFβ. IL-$10^{-/-}$ receiving SD DNA had a trend towards decreased colonic IL-2, IL-4, IL-12p35/40, TNFα, and IFNγ, with a trend towards increased TGFβ levels.

Although these data did not reach statistical significance due to the large variability between mice, these trends do suggest that under inflammatory conditions, the gut may respond differently to bacterial DNA. Further studies involving either a longer treatment time or an increased sample size are required in order to draw any conclusions regarding the effect of *in vivo* bacterial DNA treatment on colonic cytokines.

Group	Treatment	Cytokine						
		IL-2 (pg/mg)	IL-4 (pg/mg)	IL-12 p35/p40 (pg/mg)	TNFα (pg/mg)	IFNγ (pg/mg)	TGFβ (pg/mg)	IL-17a (pg/mg)
Wild-type	**Vehicle**	1327.1 ± 234.9	388.7 ± 74.5	622.0 ± 195.0	549.4 ± 89.0	125.6 ± 26.2	5459.4 ± 2323.4	15693.3 ± 3042.0
	BB DNA	1651.7 ± 181.3	622.3 ± 94.7	1212.3 ± 501.6	584.4 ± 62.8	153.8 ± 18.4	2445.3 ± 1248.2	15722.6 ± 2336.3
	SD DNA	1596.6 ± 291.2	476.1 ± 75.7	1905.0 ± 383.3	606.9 ± 83.1	114.9 ± 22.7	11625.5 ± 4910.8	16124.9 ± 2026.7
IL-10$^{-/-}$	**Vehicle**	3052.34 ± 689.3	857.6 ± 131.2[a]	18392.0 ± 6180.7[a]	804.6 ± 131.2	312.8 ± 71.3	4424.1 ± 1421.5	22480.5 ± 5296.8
	BB DNA	2237.5 ± 191.0	586.9 ± 94.1	19575.0 ± 3770.62[a]	840.8 ± 53.3[a]	290.8 ± 17.8[a]	24493.7 ± 4936.7[a]	25512.4 ± 1848.0[a]
	SD DNA	1604.3 ± 194.1	426.6 ± 83.1	6461.0 ± 1650.0[a]	563.0 ± 52.3	210.1 ± 29.4[a]	19618.2 ± 2263.1[a]	18722.0 ± 1872.7

a: P<0.05 as compared to wild type

Table 5-2 Colonic cytokines in wild-type and IL-10$^{-/-}$ mice treated with bacterial DNA. Tissue cytokines were measured in homogenized tissue with Luminex (IL-2, IL-4, IL-12p35/40, TNFα, IFNγ) or ELISA (TGFβ, IL-17a) after 7 days of treatment with bacterial DNA (BB or SD: 50µg/day).

n=5 for control. n=6 for BB DNA and SD DNA treatments

Group	*Treatment*	*Cytokine*						
		IL-2	**IL-4**	**IL-12**	**TNFα**	**IFNγ**	**TGFβ**	**IL-17a**
IL-10$^{-/-}$	**BB DNA**	-1.4	-1.5	1.1	1.0	-1.1	5.5	1.1
	SD DNA	-1.9	-2.0	-2.8	-1.4	-1.5	4.4	-1.2

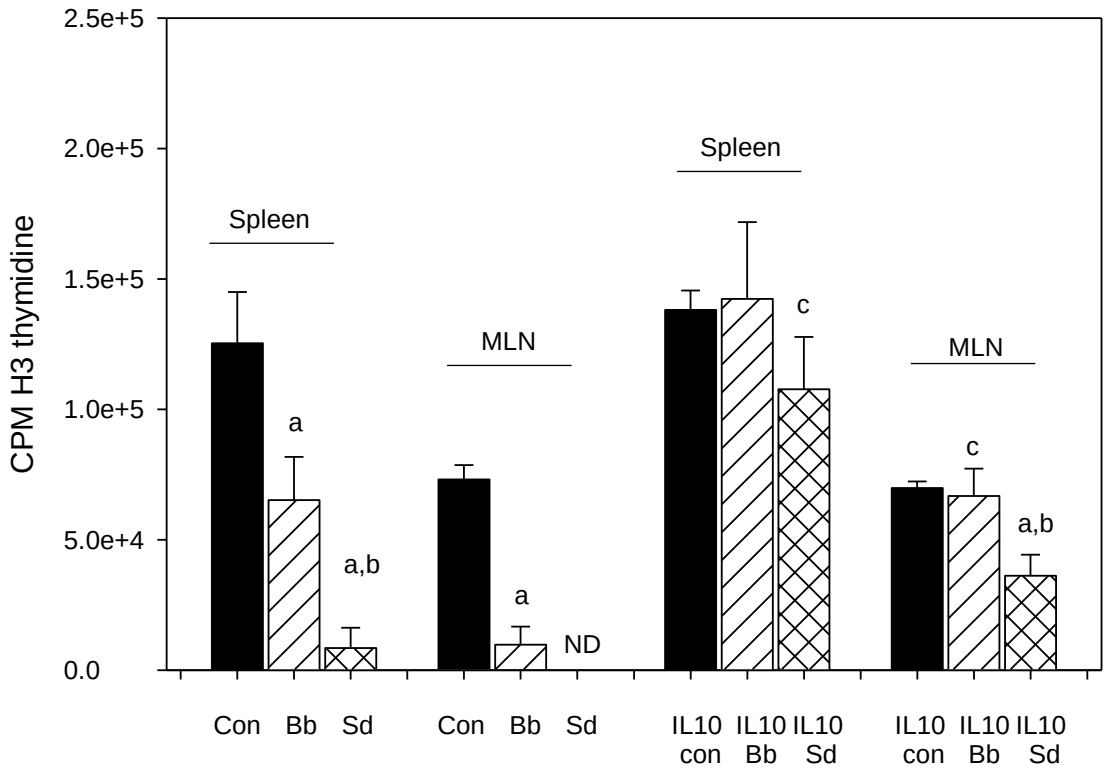
Table 5-3 Average fold difference compared to vehicle treatment. Fold change in tissue cytokines as detected by Luminex (IL-2, IL-4, IL-12p35/40, TNFα, IFNγ) or ELISA (TGFβ, IL-17a) as compared to vehicle treated mice, from colon of mice fed oral 50ug/day bacterial DNA (BB or SD) for 7 consecutive days.

n=5 for control. n=6 for BB DNA and SD DNA treatments

5-3-4 Bacterial DNA inhibits $CD4^+$ Cell proliferation in an IL-10 dependant manner

To look specifically at the ability for oral DNA treatment to modulate the $CD4^+$ T-cell activity in secondary lymphoid organs, the spleen and MLN from treated WT and IL-$10^{-/-}$ mice were harvested and $CD4^+$ cells isolated and cultured for 48hrs. Proliferation, cytokine secretion, and cellular phenotype were then analyzed.

In WT mice, oral administration of either bacterial DNA resulted in decreased $CD4^+$ cell proliferation from both the spleen and the MLN (Figure 5-4 pg 121), with SD DNA inducing a greater reduction than did BB DNA treatment. We were unable to detect any proliferation from $CD4^+$ cells isolated from the MLN of SD DNA treated mice where BB DNA was able to significantly reduce proliferation (Figure 5-4 pg 121). The ability of oral DNA treatment to reduce $CD4^+$ proliferation from the spleen and MLN was lost in the BB DNA treated IL-$10^{-/-}$ mouse where SD DNA treatment induced a significant reduction in the MLN. This suggests that the ability of bacterial DNA to reduce $CD4^+$ T cell proliferation may require the presence of IL-10, and suggests a potential role for the induction of IL-10 secreting Treg cells in response to bacterial DNA treatment.



ND= not detected

a: P<0.05 as compared to control
b: P<0.04 as compared to BB treatment
c: P< 0.03 as compared to WT treatment

Figure 5-4. Bacterial DNA inhibits $CD4^+$ cell proliferation in WT, but not in IL-$10^{-/-}$ mice. Data is given as **[**H^3] thymidine incorporation in $CD4^+$ T-cell cultures after 72 hrs expressed in counts per minute.

n=5 for control n=6 for BB DNA and SD DNA

5-3-5 Probiotic DNA induces Splenic Treg populations *in vivo*

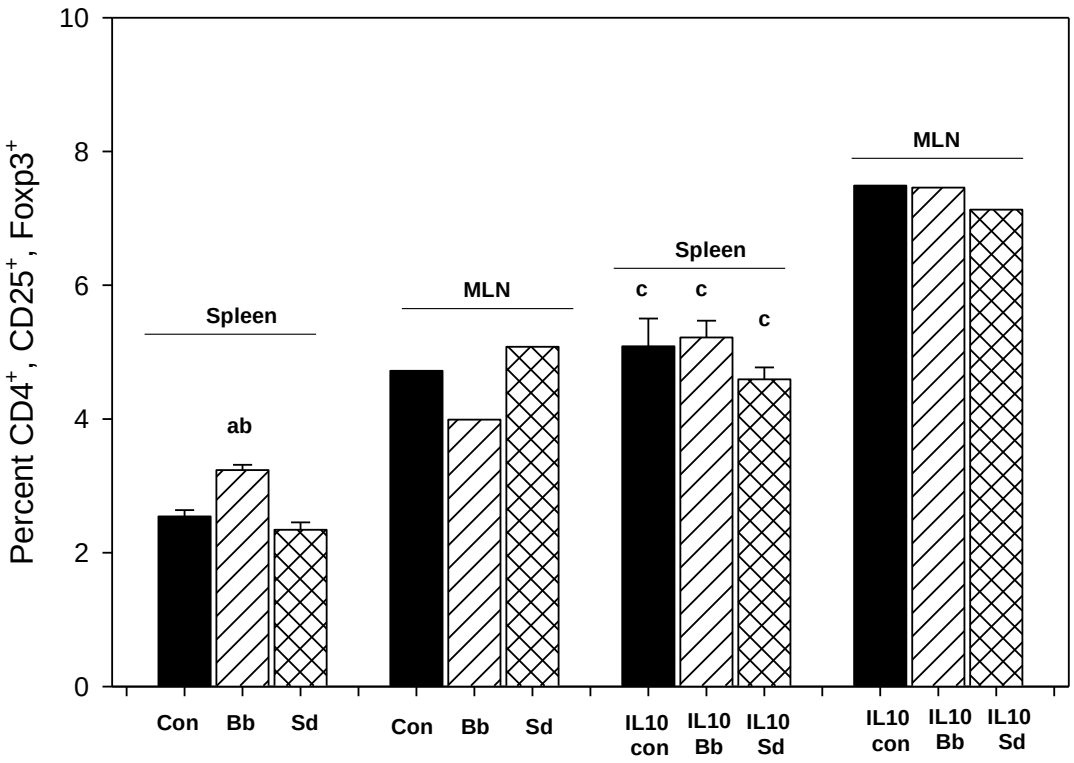
To analyze the role of bacterial DNA in inducing Treg population's *in vivo* $CD4^+$ T-cell populations were stained and analyzed for $CD25^+$, $Foxp3^+$ by flow cytometry. In WT mice, BB DNA increased Treg populations in the spleen, but not in MLN, as compared to SD and vehicle treatments (Figure 5-5 pg 124). In contrast, while IL-$10^{-/-}$ mice had increased amounts of Treg cells in both the spleen and MLN compared with WT mice, bacterial DNA did not alter the levels in either the spleen or MLN.

TGFβ and IL-10 are strong modulators of the immune response and are secreted from T-reg cells.[21] In our system, IL-10 was not detected in any of the cultures by the methods used. In WT mice, there were no significant differences in TGFβ secretion by $CD4^+$ cells isolated from the spleen of vehicle and bacterial treated mice (Table 5-4 pg 126). In IL-$10^{-/-}$ mice, levels of TGFβ secreted from CD4+ cells isolated from the spleen were significantly reduced in vehicle and SD DNA treated mice compared with levels seen in WT animals. In $CD4^+$T cells isolated from the spleens of IL-$10^{-/-}$ mice treated with BB DNA, levels of TGFβ secretion were comparable to those seen in WT mice.

There is an inverse relation between inducible Treg and Th17 cells due to the ability of both cell lineages requirement of TGFβ. [22] Naive $CD4^+$ cells will become either Tregs or Th17 dependant on the additional presence of IL-6.[22] Isolated $CD4^+$ cell preparations were analyzed by flow cytometry for the presence of $CD4^+$, IL-17^+ populations (Figure 5-6 pg 125). Although there was a

trend towards increased levels of $CD4^{+,}$ $IL\text{-}17^{+}$ cells in $IL\text{-}10^{-/-}$ mice, these values were not statistically significant due to the large degree of variability between animals. In WT mice, there was a trend towards increased levels of $CD4^{+}$, $IL\text{-}17^{+}$ in the spleen of mice treated with either BB or SD DNA, while the opposite was seen in the MLN; that being, a trend towards a decreased $CD4^{+}$, $IL\text{-}17^{+}$ population. In $IL\text{-}10^{-/-}$ mice, there was no effect of bacterial DNA treatment on levels of $CD4^{+}$, $IL\text{-}17^{+}$ T cells in the spleen. A similar reduction to that seen in WT mice in MLN levels of $CD4^{+}$, $IL\text{-}17^{+}$ cells in $IL\text{-}10^{-/-}$ mice treated with bacterial DNA was seen. No difference in IL-17a secretion from the spleen between treatments in WT mice. $IL10^{-/-}$ mice had a significant increase in splenic IL-17a secretion compared to WT with all treatments (Table 5-4 pg 126), this is concurrent with the current knowledge that IL-10 suppresses IL17a, and Th17+ Tcells[23]

In MLN of both WT and $IL\text{-}10^{-/-}$ mice, there was a trend towards increased IL-17a secretion in mice treated with BB DNA (Table 5-4 pg 126); although flow cytometry analysis appeared to indicate a reduction in Th17 cells (Figure 5-6 pg 125). In that several mice had to be combined for these MLN studies and due to the variability between mice, increasing the number of mice may resolve these discrepancies. In addition, it is possible that technical limitations precluded our accurate detection of Th17 cells in that Th17 cells may not have been maintaining their intracellular pools of IL-17a for detection by flow cytometry. The use of a Golgi inhibitor to maintain intracellular pools of cytokines before flow detection of this cell type may have resulted in different values.



a: P=0.001 as compared to Control
b: P=0.004 as compared to SD Treatment
c: P<0.002 as compared to Wild Type Treatment

Figure 5-5 Probiotic DNA increases Treg populations *in vivo*. Percent CD4+, CD25+, Foxp3+ T-cells as detected by flow cytometry from 10 week old mice fed orally 50ug/day BB or SD DNA. CD4+ cells were incubated in anti-CD3 coated plates for 48hrs and isolated for flow cytometry.

n=5 for control n=6 for BB DNA and SD DNA

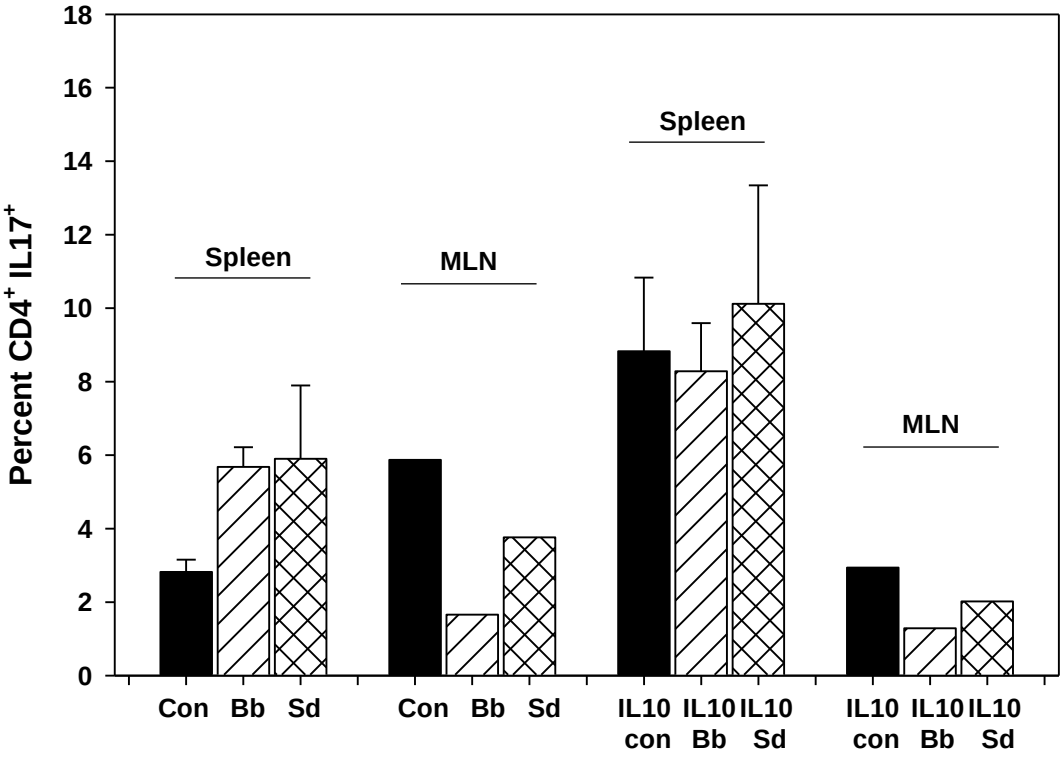


Figure 5-6 Probiotic DNA decreases MLN Th17 populations *in vivo*. Percent CD4+, IL-17+ T-cells as detected by flow cytometry from 10 week old mice fed orally 50ug/day BB or SD DNA. CD4+ cells were incubated in anti-CD3 coated plates for 48hrs and isolated for flow cytometry.

n=5 for control n=6 for BB DNA and SD DNA

			Cytokine	
Tissue	**Group**	**Treatment**	**TGFβ (pg/ml)**	**IL-17a (pg/ml)**
Spleen	**WT**	**Con**	218.8 ± 12.4	180.0 ± 21.4
		BB DNA	184.4 ± 15.3	267.2 ± 44.7
		SD DNA	229.2 ± 7.5	138.8 ± 18.6
	IL-10-/-	**Con**	130.8 ± 27.1**a**	6205.9 ± 931.5[a]
		BB DNA	179.9 ± 20.5	5325.2 ± 1121.7[a]
		SD DNA	144.5 ± 17.6**a**	4505.6 ± 804.25[a]
MLN	**WT**	**Con**	149.3	ND
		BB DNA	158.8	62.3
		SD DNA	157.2	34.1
	IL-10-/-	**Con**	179.1	588.9
		BB DNA	158.8	825.5
		SD DNA	ND	480.7

a: P<0.05 as compared to wild type

Table 5-4. TGFβ and IL-17a secretion in isolated CD4+ cells. Cytokine secretion after 48 hrs of culture as detected by ELISA from CD4+ cells isolated from WT and IL-10$^{-/-}$ mice treated with BB or SD DNA.

n=5 for control n=6 for BB DNA and SD DNA

5-4 Discussion

Here we have shown that probiotic DNA is able to maintain a less inflammatory profile in APC's during *in vitro* co-culture models of metabolic stress and inflammation, suggesting probiotic DNA may be an effective treatment for inflammatory bowel disease. Due to the variability between mice, this ability was not detected under *in vivo* conditions, as there did not appear to be any significant difference in wild type or IL-$10^{-/-}$ colonic tissue homogenates as a result of bacterial DNA treatments. However, because trends were apparent, further experiments with higher doses and/or longer treatment time may enhance these differences to statistical levels. Indeed, our data showed that probiotic DNA treatment *in vivo* was able to induce a higher population of Treg cells in the spleen compared to pathogenic bacteria DNA, possibly in an IL-10 dependant manner, suggesting a role for probiotic DNA in modulating immune function *in vivo*. The non differential response to oral administration of bacterial DNA may be due to other factors. The dose and duration of treatment may be too low to see any differences in treatment. Further, the age and number of mice could have confounding effects on the results. IL-10 deficient mice show signs of disease starting at 12 weeks of age. Extending treatment until detection of disease may enable us to determine if probiotic DNA is able to ameliorate disease and we may be able to see differences between treatments.

We previously saw that pathogenic bacteria DNA was able to induce highly inflammatory IL-17a secreting $CD4^{+}$ cells *in vitro* where BB DNA was not. However, these same results were not seen *in vivo.* Although a small trend

towards decreased Th17 cells was detected in MLN with probiotic DNA treatment in both the wild type and IL-10$^{-/-}$ mouse, the opposite was seen in IL-17a secretion. Further, there were no differences detected in splenic levels of Th17 cells in wild type mice as we had expected to see based upon results from the *in vitro* tri culture system. Again, technical limitations in accurately detecting Th17 cells may have altered these results. Further, it is possible that the bacterial DNA did not reach the colon in the *in vivo* experiments, and thus was unable to have a direct effect as was examined in the *in vitro* experiments.

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Chapter 6 Conclusions and discussions

6.1 Overview of the project

Inflammatory bowel diseases are complex inflammatory diseases with unknown etiology. IBD affects an ever increasing population of the western world including Canada and the US.[1] IBD is a multi-allelic disease that results in a dysregulated immune response to the host microflora. The outside luminal microflora and the mucosal immune system of the host are separated by a single cell layer of intestinal epithelial cells. The mucosal immune system is a nexus of innate and adaptive immunity that must be managed to maintain homeostasis. The role of intestinal epithelial cells in maintaining this homeostasis is only now being understood. There is evidence that IEC's have the ability to modulate the mucosal immune system through secreted factors[2] and antigen presentation through exosomal vesicles[3]. IEC's primarily see the world through innate immune receptors including toll like receptors. Of these receptors TLR9 is associated with gut homeostasis where the loss of TLR9 results in exacerbated chemically induced disease and is required for the anti-inflammatory effects of probiotics.[4] TLR9's natural ligand is microbial DNA, specifically the immune-stimulatory unmethylated CpG motifs found within the DNA. IEC's express apical cell surface TLR9 and are able to differentiate between bacterial species by sensing their DNA.[5-6] Further, gut flora DNA has the ability to modulate T regulatory and T effector cell levels in the gut.[7] It is the combination of these observations that led us to look at the role IEC's play in modulating the immune

activity in response to bacteria DNA. This research gives further insight into the dominant role IEC's play in immune response regulation, and suggests a possible mechanism by which probiotics exert their anti-inflammatory effects.

6.2 Project conclusions

In this thesis I have shown that intestinal epithelial cells have the ability to modulate both adaptive and innate responses through interactions with bacterial DNA. IEC's are able to do this through differential gene expression, resulting in secreted factors and direct interactions with APC's. It is partially through these mechanisms that the mucosal immune system is able to identify between commensal microflora, probiotics, and pathogens within the luminal contents, and initiate the appropriate immune response to the diverse microbial load. DNA isolated from a probiotic strain results in an IEC dependant "tolerant" immune response from APC's as determined by reduced cytokine secretion. Further probiotic bacteria DNA is able to maintain this profile during inflammation and metabolic stress, both associated with inflammatory disease state, suggesting that DNA from *Biffidobacteria* based probiotics is an active and safe component of probiotic treatment. Probiotic DNA treatment also results in a downstream decrease in Th17 effector T-cell *in vitro* in the presence of IEC's. *In vivo* we see that both DNA as an oral administration are able to decrease CD4+ T cell proliferation. However, probiotic DNA is also able to increase systemic Treg populations as detected in the spleen. This ability is lost in the IL-$10^{-/-}$ mouse suggesting a strong role for IL-10 in this mechanism. Further, a trend toward decreased Th17 cells isolated from the MLN of wild type and $IL10^{-/-}$ mice orally

fed BB DNA but not SD DNA mimics our *in vitro* data. An increased number of mice may result in a significant response and needs to be done to determine if this is true. Overall this data suggests that probiotic DNA initiates its anti-inflammatory actions in an IEC dependant manner by decreasing the inflammatory Th17 population.

Intestinal epithelial cells are a barrier between a huge antigenic load, the lumenal milieu, and a highly active immune organ. They play a very active role in modulating the mucosal immune response to bacterial DNA through their own differential cellular response, secreted factors such as TSLP that modulate the environmental milieu, and direct interaction with antigen presenting cells. Human and mouse intestinal epithelial cells have the ability to differentiate between DNA isolated from different bacteria species. This is not only detected by secretion of IL-8[5-6] and KC-1 (Figure 4-2 pg 83), but a myriad of secreted cytokines (Table 4-1 pg 79) and genes as seen in chapter 3. The extent to which IEC's are able to differentiate between bacteria DNA has not been shown before and was the first aim of this thesis. Further, the ability of IEC's to differentiate between bacterial species DNA results in downstream modulation of the adaptive immune system *in vitro* and *in vivo* suggesting that IEC's play a dominant role in orchestrating mucosal immunity in a TLR9 dependant manner. The mechanism by which IEC's do this is a question that has yet to be answered.

6.3 Research Limitations

The use of *in vitro* and *in vivo* models of disease often has limitations to their application in human disease. Animal and *in vitro* models are required tools for medical research allowing us to test treatments, increase our knowledge of mechanism and physiology. These tools give researchers the ability to simplify a system and isolate the players to determine unique function and mechanisms of action. However, we must always acknowledge that these reductionist models are just that; models and what is truly happening in human disease requires clinical research. Of these research models *in vitro* models have the most inherent limitations. We utilized a multi cell culture system adapted to mimic the mucosal immune system (Figure 2-2 pg 37). The major cell types implicated in this model are intestinal epithelial cells, antigen presenting cells and CD4+ T-cells. I will briefly discuss the limitations of each cell type *in vitro*.

6-3-1 Intestinal Epithelial Cells

Most significant is the origin and alteration of the intestinal epithelial cells. MODE-K cells were derived through transfection with SV40 large T cell gene. This has multiple implications most notably being the cells ability to be immortalized and grow in culture.[8] I used these cells partially for this ability as isolation of primary IEC's from mice or human tissue is very difficult and there are no "standard" protocols that have achieved this. Preparations of primary IEC's often suffer from mass cell apoptosis induced by loss of cell anchorage (anoikis).[9] Further, the access to human intestinal explants is limited, requiring

the use of transformed and cancer cell lines for basic research. The transformed MODE-K cell line expresses T cell MHCII when activated with IFNγ making them an artificial APC with the ability to differentiate naïve T-cells.[10] Due to this unique ability of IEC's and the fact that there are mismatched MHCII between the cell line and the 129Sv/ev mouse model, $CD4^+$ T cells were never in direct contact with the MODE-K cells. This cellular isolation mimicked the anatomy of the mucosal immune system with the main pool of naive T-cells residing in the MLN and not in close contact with the intestinal barrier. The human intestinal epithelial cells, HT29, employed in the experiments here are a cancer cell line. Cancer cells are cells that proliferate in an unregulated manner. This is due to various changes in intracellular pathways that are not completely understood. Irregardless they are less susceptible to apoptosis and can be grown indefinitely. It is because of these transformations that the cellular response and even the receptors that are expressed may not be the same as primary derived IEC's.

6-3-2 Bone Marrow Derived APC's-

These cells are cultured from hematopoietic cells from bone marrow. As discussed in Chapter 2 they do not have a definitive cellular phenotype that matched those ones isolated *in vivo*. The use of these cells is due to the vast numbers that can be cultured compared to those that can be harvested in the blood or lamina propria. Due to their different phenotype the information gained from using them is not directly comparable to *in vivo* populations. These cells do however allow us to discern the effect of co-culture in response to single ligands which is a very powerful tool in understanding cellular physiology. Further

studies using human peripheral blood mononuclear cell isolations of APC's and T-cells need to be done to validate the effects seen in mice. This is not only due to the difference in the derivation of the APC's but also that the murine and human immune systems have differences.

6-3-3 CD4+ T cells

T-cells isolated from the spleen and MLN are often used in scientific research. These cells are easy to isolate and function in much the same way as those *in vivo*. In this model these cells and mice are not primed to a specific protein antigen such as OVA; thus we artificially activated them using beads coated with antibodies against CD28 and CD3. This induced both signals required by $CD4^+$ T-cells to activate as if they were seeing a properly loaded cognate MHCII receptor. This allows us to look at the innate immune activation without protein antigen. Again, as with all artificial models there could be some discrepancy compared to those models where activation is through the proper physiological mechanism. Although our *in vitro* tri culture system is a reductionist and in some ways artificial system it allows us to look at each specific cell type separately and in conjunction with each other making it a powerful tool to observe these interactions under strictly controlled conditions which cannot be controlled *in vivo*.

6-3-4 Animal models

Animal models are indispensible to the study of human disease. There are a multitude of animal models used to study IBD although none show the exact

same symptoms as human disease. This highlights the complexity that is involved in disease as well as the limitations of using *in vivo* models. Of the mouse models currently used, three main physiological areas are affected; chemical induced models change the integrity and permeability of the epithelial barrier, innate immune dysregulation and adaptive immune dysregulation. The IL10-/- model used here is a well studied model of spontaneous. This model most closely resembles human Crohn's disease and requires colonic microbiota to occur.[11] Due to the lack of regulatory $IL10^{-/-}$ there are increased inflammatory mediators and cytokines detected in the colon, resulting in an unregulated immune response to the resident microbial flora.[12] One of the obvious limitations in using a mouse model to understand immunological activity is the varying discrepancies between the human and the mouse adaptive immune system.

There are these limitations in all research areas and should be taken into account at all times. Although the models are not perfect, they afford us the ability to do research and have given us much of the insight we have today into human disorders.

6.4 Discussion and Future Research

Often the more we know, the more there is to know. Research often results with more questions than answers. Same can be said for the data presented in this thesis. One of the primary questions needed to be addressed is the ability of IEC's to modulate downstream T-cell activity during inflammation

and more work needs to be done to address the role of bacteria DNA *in vivo*. This thesis gives us hints towards what may be happening but further studies must be done to definitively answer this question. Further study has to be done to determine if bacterial DNA is a viable treatment option by extending treatment time and examining the disease response *in vivo*. On a larger scale the differences between bacterial DNA and how intestinal epithelial cells are able to differentiate between bacteria DNA in a species specific manner is currently unknown. As discussed in chapter 4 it may partially due to the location of DNA sensing TLR9 potentially a cell surface co-receptor like the MD1 receptor associated with TLR4. It could be purely due to different amounts of stimulatory to non stimulatory motifs in each bacterial DNA species or the level of methylation may have an effect on its ability to activate TLR9. Beyond the IEC's themselves, are questions about the exact mechanism by which IEC's modulate immune activity, which secreted factors are relevant, how are the IEC's interacting directly with APC's, what changes are induced in APC's to make them less immunogenic. Is it through secreted factors or direct interaction that these APC's are able to interact with CD4+ T-cell? Is this a CD4+ cell specific modulation or do they have effects on CD8+ T-cell population? Is this modulation effective in human immune models? Irregardless of the number of questions, there is much work to be done to understand the role IEC's play in bridging the innate and adaptive immune system to maintain homeostasis. Further understanding will allow us to identify disease pathology and new targets for disease treatment.

IBD is a complex set of diseases the study of which requires knowledge from a variety of specialties including genetics, human physiology, immunology and microbiology. Within these specialties you can find exciting and novel areas to study. It is also due to the complexity of this disease that multidisciplinary research is needed to advance our knowledge and help treat disease. Classically, intestinal epithelial cells were seen as a physiological barrier between the sterile inside of the host and the outside environment the lumen. It is through decades of research that the dynamic role of IEC's is understood in nutritional uptake and mucosal immunity. This thesis further expands our current knowledge of IEC's as immune modulators where they act to bridge both the innate and adaptive immunity to maintain host tolerance. Due to the complexity of the system and the multiple factors associated with it, there are more questions that have yet to be answered than have been answered by my contribution. It is here that we look toward the future of research to greater understand the complex interrelated systems associated with health and hopefully treatment of disease.

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Appendix 1: Human Immune Panel - Taqman Low Density Array

Information from gene search- www.genecards.com

Cytokines

IL-1A, IL-1B - Cytokine- Secreted by activated monocytes stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity.

IL-2 - Cytokine- Produced by T-cells in response to antigenic or mitogenic stimulation, this protein is required for T-cell proliferation and other activities crucial to regulation of the immune response. (Th1)

IL-3 - Cytokine- A growth promoting cytokine. This CSF induces granulocytes, macrophages, mast cells, stem cells, erythroid cells, eosinophils and megakaryocytes

IL-4 - Cytokine- Participates in at least several B-cell activation processes as well as of other cell types. It is a co-stimulator of DNA-synthesis. It induces the expression of class II MHC molecules on resting B-cells. It enhances both secretion and cell surface expression of IgE and IgG1. (Th2)

IL5 - Cytokine- Factor that induces terminal differentiation of late-developing B-cells to immunoglobulin secreting cells. (Th2)

IL-6 - Cytokine-plays an essential role in the final differentiation of B-cells into Ig-secreting cells, it induces myeloma and plasmacytoma growth, it induces nerve cells differentiation; in hepatocytes it induces acute phase reactants. Required for Th17 cell differentiation [1]. (Th17)

IL-7 - Cytokine- Hematopoietic growth factor capable of stimulating the proliferation of lymphoid progenitors. It is important for proliferation during certain stages of B-cell maturation.

IL-8 - Cytokine- Chemotactic factor that attracts neutrophils, basophils, and T-cells, but not monocytes. It is also involved in neutrophil activation. It is released from several cell types, including intestinal epithelial cells [2] in response to an inflammatory stimulus.

IL-9 - Cytokine- Supports IL-2 independent and IL-4 independent growth of helper T-cells. This cytokine stimulates Proliferation and Apoptosis.

IL-10 – Cytokine- Inhibits the synthesis of a number of cytokines, including IFNγ, IL-2, IL-3, TNFα and GM-CSF produced by activated macrophages and by helper T-cells. (Treg)

IL-12A – Cytokine- Associates with IL-12B cytokine to produce and active heterodimer that can act as a growth factor for activated T and NK cells, enhance the lytic activity of NK/lymphokine-activated Killer cells, and stimulate the production of IFN-gamma by resting Peripheral Blood mononuclear cells. IL-12p35 subunit. (Th1)

IL-12B – Cytokine- Associates with either IL-12A to produce IL-12 heterodimer or IL-23A to form the IL-23 interleukin, and heterodimeric cytokine which functions in innate and adaptive immunity. IL-23 may constitute with IL-17 an acute response to infection in peripheral tissues. IL-23 induces autoimmune inflammation and thus may be responsible for autoimmune inflammatory diseases and may be important for tumorigenesis. IL-12p40 subunit (Th1, or Th17)

IL-13- Cytokine - Inhibits inflammatory cytokine production. Synergizes with IL2 in regulating IFNγ synthesis. May be critical in regulating inflammatory and immune responses. (Th2)

IL-15- Cytokine- Cytokine that stimulates the proliferation of T-lymphocytes. IL-15 and IL-2 are found to bind common hematopoietin receptor subunits, and may compete for the same receptor, and thus negatively regulate each other's activity. The number of CD8+ memory cells is shown to be controlled by a balance between this cytokine and IL-2. (Th1)[3]

IL-17- Cytokine- Induces stromal cells to produce proinflammatory and hematopoietic cytokines. Enhances the surface expression of the intracellular adhesion molecule-1 (ICAM-1) in fibroblasts.

IL-18- Cytokine- Augments natural killer cell activity in spleen cells and stimulates IFNγ production in T-helper type I cells. Stabilizes Th1 cell differentiation. (Th1)[3]

IFNγ- Cytokine- Produced by lymphocytes activated by specific antigens or mitogens. IFN-gamma, in addition to having antiviral activity, has important immunoregulatory functions. It is a potent activator of macrophages, it has antiproliferative effects on transformed cells and it can potentiate the antiviral and antitumor affects of the type I interferons. (Th1)

TNF- Cytokine- Binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion and is implicated in the induction of cachexia, under certain conditions it can stimulate cell proliferation and induce cell differentiation.

TGFb1- Cytokine- Multifunctional protein that control proliferation, differentiation, and other functions in many cell types. Many cells synthesize TGFB1 and essentially all of them have specific receptors for this protein. It regulates the actions of many other growth factors and determines a positive or negative direction of their effects. (Treg, Th17)

LTA- Cytokine- In its homotrimeric form binds to TNFRSF1A/TNFR1, TNFRSF1B/TNFBR and TNFRSF14/HVEM. In its heterotrimeric form with LTB binds to TNFRSF3/LTBR. Lymphotoxin is produced by lymphocytes and cytotoxic for a wide range of tumor cells in vitro and in vivo.

CSF1- Cytokine - Induces cells of the monocyte/macrophage lineage. It plays a role in immunological defenses, bone metabolism, lipoproteins clearance, fertility and pregnancy.

CSF2- Cytokine - stimulates the growth and differentiation of hematopoietic precursor cells from various lineages, including granulocytes, macrophages, eosinophils and erythrocytes

CSF3- Cytokine - This CSF induces granulocytes.

Chemokine

CCL3- Chemokine- Monokine with inflammatory and chemokinetic properties. Binds to CCR1, CCR4 and CCR5. One of the major HIV-suppressive factors produced by CD8+ T-cells.

CCL19- Chemokine- May play a role not only in inflammatory and immunological responses but also in normal lymphocyte recirculation and homing. May play an important role in trafficking of T-cells in thymus, and T-cell and B-cell migration to secondary lymphoid organs. Specifically binds to chemokine receptor CCR7.

CCL2- Chemokine- Chemotactic factor that attracts monocytes and basophils but not neutrophils or eosinophils. Augments monocyte anti-tumor activity. Has been implicated in the pathogenesis of diseases characterized by monocytic infiltrates, like psoriasis, rheumatoid arthritis or atherosclerosis.

CCL5- Chemokine- Chemoattractant for blood monocytes, memory T-helper cells and eosinophils. Causes the release of histamine from basophils and activates eosinophils.

CCR2- Chemokine Receptor- Receptor for the MCP-1, MCP-3 and MCP-4 chemokines. Transduces a signal by increasing the intracellular calcium ions level. Alternative co-receptor with CD4 for HIV-1 infection.\

CCR4- Chemokine Receptor- High affinity receptor for the C-C type chemokines CCL17/TARC and CCL22/MDC. The activity of this receptor is mediated

by G(i) proteins which activate a phosphatidylinositol-calcium second messenger system.

CCR5- Chemokine receptor- Receptor for a number of inflammatory CC-chemokines including MIP-1-alpha, MIP-1-beta and RANTES and subsequently transduces a signal by increasing the intracellular calcium ion level.

CCR7- Chemokine Receptor - Receptor for the MIP-3-beta chemokine. Probable mediator of Epstein bar virus effects on B-lymphocytes or of normal lymphocyte functions.

CXCR3- Chemokine receptor- Receptor for CXCL9, CXCL10 and CXCL11 and mediates the proliferation of human mesangial cells (HMC).

CXCL10- Chemokine- Chemotactic for monocytes and T-lymphocytes. Binds to CXCR3

CXCL11- Chemokine- Chemotactic for interleukin-activated T-cells but not unstimulated T-cells, neutrophils or monocytes. Induces calcium release in activated T-cells. Binds to CXCR3. May play an important role in CNS diseases which involve T-cell recruitment.

Secreted Factor

NOS2A- Secreted factor- Produces nitric oxide (NO) which is a messenger molecule with diverse functions throughout the body. In macrophages, NO mediates tumoricidal and bactericidal actions.

VEGF- Secreted Factor- Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis, and induces permeabilization of blood vessels.

ICOS- Secreted Factor- Enhances all basic T-cell responses to a foreign antigen, namely proliferation, secretion of lymphokines, up-regulation of molecules that mediate cell-cell interaction, and effective help for antibody secretion by B-cells. Essential both for efficient interaction between T and B-cells and for normal antibody responses to T-cell dependent antigens. Does not up-regulate the production of interleukin-2, but super induces the synthesis of interleukin-10. Prevents the apoptosis of pre-activated T-cells. Plays a critical role in CD40-mediated class switching of immunoglobin isotypes

Cellular Markers

CD3E- Cellular Marker- CD3-epsilon polypeptide, which together with CD3-gamma, -delta and -zeta, and the T-cell receptor alpha/beta and gamma/delta heterodimers, forms the T-cell receptor-CD3 complex. This complex plays an important role in coupling antigen recognition to several intracellular signal-transduction pathways.

CD4- Cellular Marker- Accessory protein for MHC class-II antigen/T-cell receptor interaction. May regulate T-cell activation. Induces the aggregation of lipid rafts.

CD8a- Cellular Marker- cell surface glycoprotein found on most cytotoxic T lymphocytes that mediates efficient cell-cell interactions within the immune system. The CD8 antigen, acting as a co-receptor, and the T-cell receptor on the T lymphocyte recognize antigen displayed by an antigen presenting cell (APC) in the context of class I MHC molecules.

CD19- Cellular Marker- Assembles with the antigen receptor of B lymphocytes in order to decrease the threshold for antigen receptor-dependent stimulation.

IL12RA- (CD25) -Cellular Marker- The interleukin 2 (IL2) receptor alpha (IL2RA) and beta (IL2RB) chains, together with the common gamma chain (IL2RG), constitute the high-affinity IL2 receptor.

CD28- Cellular Marker- CD28 co-stimulation is essential for CD4 positive T-cell proliferation, survival, interleukin-2 production, and T-helper type-2 (Th2) development.

CD38- Cellular Marker - A novel multifunctional ectoenzyme widely expressed in cells and tissues especially in leukocytes. CD38 also functions in cell adhesion, signal transduction and calcium signaling.

CD34- Cellular marker- Possible adhesion molecule with a role in early hematopoiesis by mediating the attachment of stem cells to the bone marrow extracellular matrix or directly to stromal cells. Could act as a scaffold for the attachment of lineage specific glycans, allowing stem cells to bind to lectins expressed by stromal cells or other marrow components.

CD40- Cellular Marker - This receptor has been found to be essential in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation.

PTPRC- Cellular Marker- This gene is specifically expressed in hematopoietic cells. This PTP has been shown to be an essential regulator of T- and B-cell antigen receptor signaling. Required for T-cell activation through the antigen receptor.

CD68- Cellular Marker- A transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophages. Could play a role in

phagocytic activities of tissue macrophages, both in intracellular lysosomal metabolism and extracellular cell-cell and cell-pathogen interactions.

CD80- Cellular Marker - Involved in the co-stimulatory signal essential for T-lymphocyte activation. T-cell proliferation and cytokine production is induced by the binding of CD28 or CTLA-4 to this receptor.

CD86- Cellular marker - Receptor involved in the costimulatory signal essential for T-lymphocyte proliferation and interleukin-2 production, by binding CD28 or CTLA-4. May play a critical role in the early events of T-cell activation and costimulation of naive T-cells, such as deciding between immunity and anergy that is made by T-cells within 24 hours after activation.

CTLA4- Cellular marker- Possibly involved in T-cell activation. Binds to B7-1 (CD80) and B7-2 (CD86)

CD40LG- Cellular marker- Mediates B-cell proliferation in the absence of co-stimulus as well as IgE production in the presence of IL-4. Involved in immunoglobulin class switching.

HLA-DRA- Cellular marker- This class MHC II molecule is a heterodimer consisting of an alpha (DRA) and a beta chain (DRB), both anchored in the membrane. It plays a central role in the immune system by presenting peptides derived from extracellular proteins.

HLA-**DRB1**- Cellular marker-The class II molecule is a heterodimer consisting of an alpha (DRA) and a beta chain (DRB), both anchored in the membrane. It plays a central role in the immune system by presenting peptides derived from extracellular proteins.

TBX21- Cellular marker- Transcription factor that controls the expression of the TH1 cytokine, interferon-gamma. Initiates TH1 lineage development from naive TH precursor cells both by activating TH1 genetic programs and by repressing the opposing TH2 programs. (Th1)

TNFRSF18- Cellular marker- Receptor for TNFSF18. Seems to be involved in interactions between activated T-lymphocytes and endothelial cells and in the regulation of T-cell receptor-mediated cell death. Mediated NF-kappa-B activation via the TRAF2/NIK pathway.

SKI- Cellular marker- May play a role in terminal differentiation of skeletal muscle cells but not in the determination of cells to the myogenic lineage.

Intracellular Signaling

STAT3- Intracellular Signaling- Transcription factor that binds to the interleukin-6 (IL-6)-responsive elements identified in the promoters of various acute-phase protein genes.

NFKB2- Intracellular Signaling- NF-kappa-B is a pleiotropic transcription factor which is present in almost all cell types and is involved in many biological processed such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis.

IKBKB- Intracellular Signaling- Acts as part of the IKK complex in the conventional pathway of NF-kappa-B activation and phosphorylates inhibitors of NF-kappa-B thus leading to the dissociation of the inhibitor/NF-kappa-B complex and ultimately the degradation of the inhibitor.

SMAD3- Intracellular signaling- Transcriptional modulator activated by TGF-beta (transforming growth factor) and activin type 1 receptor kinase.

SMAD7- Intracellular signaling- antagonist of signaling by TGF-beta (transforming growth factor) type 1 receptor superfamily members; has been shown to inhibit TGF-beta (Transforming growth factor) and activin signaling by associating with their receptors thus preventing SMAD2 access.

AGTR1- Intracellular signaling- Receptor for angiotensin II. Mediates its action by association with G proteins that activate a phosphatidylinositol-calcium second messenger system.

AGTR2- Intracellular signaling- Receptor for angiotensin II. Cooperates with MTUS1 to inhibit ERK2 activation and cell proliferation.

Apoptosis

BCL2- Apoptosis- Suppresses apoptosis in a variety of cell systems including factor-dependent lymphohematopoietic and neural cells. Regulates cell death by controlling the mitochondrial membrane permeability.

BCL2L1- Apoptosis- Potent inhibitor of cell death. Isoform Bcl-X(L) anti-apoptotic activity is inhibited by association with SIVA isoform 1. Inhibits activation of caspases (By similarity). Appears to regulate cell death by blocking the voltage-dependent anion channel (VDAC) by binding to it and preventing the release of the caspase activator, cytochrome c, from the mitochondrial membrane. The Bcl-X(S) isoform promotes apoptosis

BAX- Apoptosis- Accelerates programmed cell death by binding to, and antagonizing the apoptosis repressor BCL2 or its adenovirus homolog E1B 19k protein. Induces the release of cytochrome c, activation of CASP3, and thereby apoptosis.

FAS- Apoptosis- The protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor contains a death domain. It has been shown to play a central role in the physiological regulation of programmed cell death.

FASL- Apoptosis- Interaction of FAS with this ligand is critical in triggering apoptosis of some types of cells such as lymphocytes. Defects in this gene may be related to some cases of systemic lupus erythematosus (SLE).

Enzymes

HMOX1- Enzyme- Heme oxygenase cleaves the heme ring at the alpha methene bridge to form biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Under physiological conditions, the activity of heme oxygenase is highest in the spleen, where senescent erythrocytes are sequestrated and destroyed

PTGS2- Enzyme- Prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase, is the key enzyme in prostaglandin biosynthesis. May have a role as a major mediator of inflammation and/or a role for prostanoid signaling in activity-dependent plasticity.

RPL3L- Enzyme- Unlike the ubiquitous expression of ribosomal protein genes, this gene has a tissue-specific pattern of expression, with the highest levels of expression in skeletal muscle and heart. It is not currently known whether the encoded protein is a functional ribosomal protein or whether it has evolved a function that is independent of the ribosome.

CYP1A2- Enzyme- Cytochromes P450 is a group of heme-thiolate mono-oxygenases. In liver microsomes, this enzyme is involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics.

CYP7A1- Enzyme- Cytochromes P450 is a group of heme-thiolate mono-oxygenases. In liver microsomes, this enzyme is involved in an NADPH-

dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics.

REN- Enzyme- Renin catalyzes the first step in the activation pathway of angiotensinogen. Play a role as a marker and a regulator of neuronal differentiation; Up-regulated by a variety of neurogenic signals, such as retinoic acid, epidermal growth factor/EGF and NGFB/nerve growth factor. Induces apoptosis, growth arrest and the expression of cyclin-dependent kinase inhibitor CDKN1B.

ACE- Enzyme- Converts angiotensin I to angiotensin II by release of the terminal His-Leu, this results in an increase of the vasoconstrictor activity of angiotensin. Also able to inactivate bradykinin, a potent vasodilator. Has also a glycosidase activity which releases GPI-anchored proteins from the membrane by cleaving the mannose linkage in the GPI moiety.

Degranulation, Compliment

PRF1- Degranulation, Compliment- In the presence of calcium, perforin polymerizes into transmembrane tubules and is capable of lysing non-specifically a variety of target cells.

GZMB- Degranulation, Compliment- This enzyme is necessary for target cell lysis in cell-mediated immune responses. It cleaves after Asp. Seems to be linked to an activation cascade of caspases (aspartate-specific cysteine proteases) responsible for apoptosis execution. Cleaves caspase-3, -7, -9 and 10 to give rise to active enzymes mediating apoptosis.

GNLY- Degranulation, Compliment- Is located in the cytotoxic granules of T cells, which are released upon antigen stimulation. Antimicrobial protein that kills intracellular pathogens. Active against a broad range of microbes, including Gram-positive and Gram-negative bacteria, fungi, and parasites. Kills *Mycobacterium tuberculosis*.

C3- Degranulation, Compliment- Derived from proteolytic degradation of complement C3, C3a anaphylatoxin is a mediator of local inflammatory process. It induces the contraction of smooth muscle, increases vascular permeability and causes histamine release from mast cells and basophilic leukocytes.

Cellular Migration

FN1- Cellular migration- Fibronectins bind cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin. Fibronectins are involved in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape. Interaction with TNR mediates inhibition of cell adhesion and neurite outgrowth.

COL4A5- Cellular migration- Type IV collagen is the major structural component of glomerular basement membranes (GBM), forming a 'chicken-wire' meshwork together with laminins, proteoglycans and entactin/nidogen.

LRP2- Cellular migration- Acts together with cubilin to mediate HDL endocytosis (By similarity). May participate in regulation of parathyroid-hormone and para-thyroid-hormone-related protein release.

ICAM1- Cellular Migration- ICAM proteins are ligands for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2). During leukocyte trans-endothelial migration, ICAM1 engagement promotes the assembly of endothelial apical cups through SGEF and RHOG activation.

SELP- Cellular Migration- Ca(2+)-dependent receptor for myeloid cells that binds to carbohydrates on neutrophils and monocytes. Mediates the interaction of activated endothelial cells or platelets with leukocytes. The ligand recognized is sialyl-Lewis X. Mediates rapid rolling of leukocyte rolling over vascular surfaces during the initial steps in inflammation through interaction with PSGL1

SELE- Cellular Migration- Cell-surface glycoprotein having a role in immune-adhesion. Mediates in the adhesion of blood neutrophils in cytokine-activated endothelium through interaction with PSGL1/SELPLG. May have a role in capillary morphogenesis.

Endogenous Controls

MYH6- Endogenous control- MYH6 myosin, heavy chain 6, cardiac muscle, alpha, function: muscle contraction.

EDN1- Endogenous Control- Endothelins are endothelium-derived vasoconstrictor peptides.

GAPDH- Endogenous control- Independent of its glycolytic activity it is also involved in membrane trafficking in the early secretory pathway.

ACTB- Endogenous control- Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.

TFRC- Endogenous control- Cellular uptake of iron occurs via receptor-mediated endocytosis of ligand-occupied transferrin receptor into specialized endosomes. Endosomal acidification leads to iron release.

GUSB- Endogenous control- Plays an important role in the degradation of dermatan and keratan sulfates.

PGK1- Endogenous Control- In addition to its role as a glycolytic enzyme, it seems that PGK-1 acts as a polymerase alpha cofactor protein.

ECE1- Endogenous Control- Converts big endothelin-1 to endothelin-1.

References

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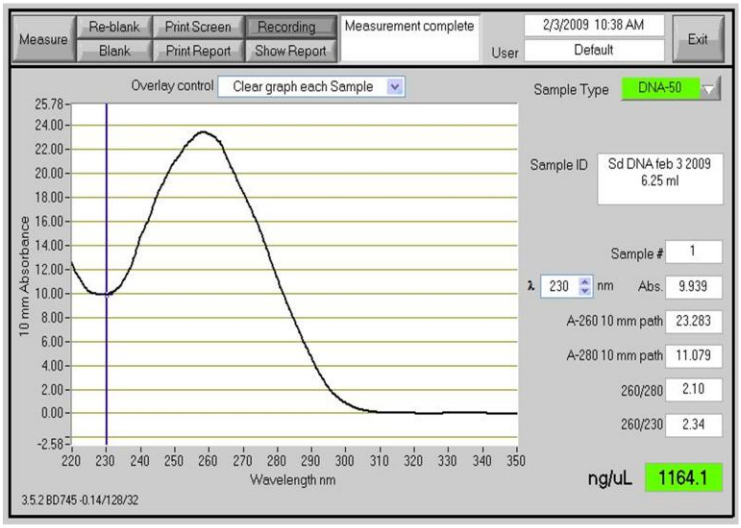
APPENDIX 2 -DNA isolation and DNAse Treatment

A



B

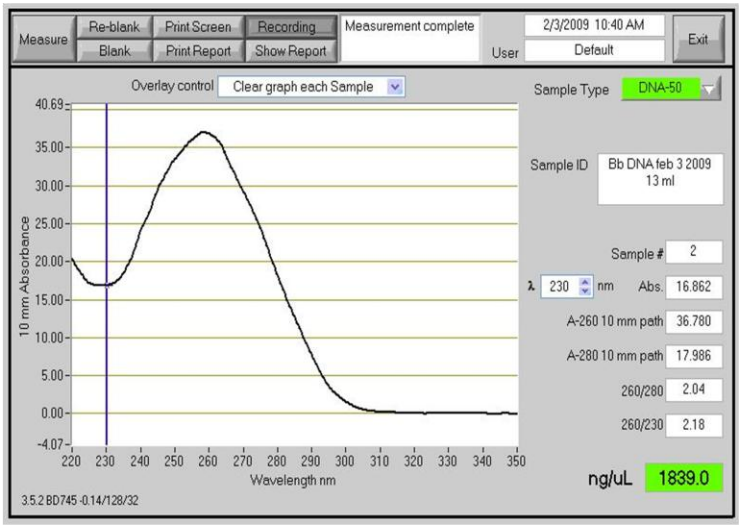
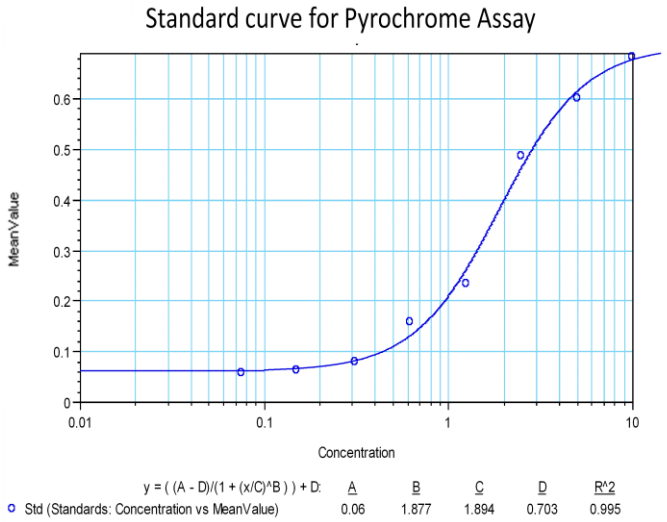


Figure 1. DNA purity and concentration determined by Nanodrop. A) SD DNA isolation from February 3, 2009. B) BB DNA isolation from February 3, 2009. Figures are representative of all analysis of DNA preparations.

A



B

Sample	Wells	Values	Result	Mean Result	Std.Dev.	CV%
S.D DNA	A7	0.602	4.625	4.523	0.097	2.1
	A8	0.595	4.433			
	A9	0.598	4.513			
B.B DNA	F7	0.62	5.222	5.301	0.31	5.8
	F8	0.615	5.038			
	F9	0.63	5.642			

Figure 2: Analysis of endotoxin Quantitation in bacterial DNA Prep. A) Std Curve of control e.coli LPS. B) Endotoxin detected expressed in ng per 50ug of DNA. Figures are representative of all analysis of DNA preparations.

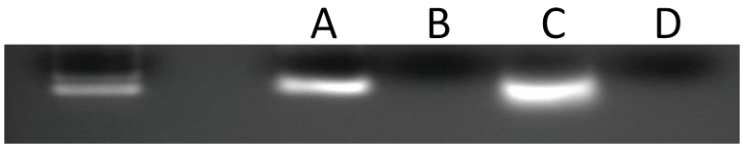


Figure 3. Analysis of DNA preparations and DNAse treatment by agarose gel electrophoresis. A) BB DNA. B)BB DNA + DNAse. C) SD DNA. D) SD DNA + DNAse. Figures are representative of all analysis of DNA preparations.