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The Role of Small Intestinal Permeability in the Pathogenesis of Colitis in the Interleukin-10 Gene Deficient Mouse.

by

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A mi esposo Esteban y a mi hija Marisol.

Abstract

The Role of Small Intestinal Permeability in the Pathogenesis of Colitis in the Interleukin-10 Gene Deficient Mouse

It is currently believed that the etiology of inflammatory bowel disease involves an aberrant immune response towards the gastrointestinal microbial flora. In addition, an increase in intestinal paracellular permeability may also be a contributing factor of disease, as it precedes disease in several animal models. However, it remains unclear whether increased intestinal permeability is an epiphenomenon of disease or if it can lead to it. The goal of this thesis is to elucidate this cause-effect relationship.

The IL-$10^{-/-}$ mouse is a model of IBD that spontaneously develops colitis after 12 weeks of age. We measured intestinal permeability in this mouse from 4-17 weeks of age and observed that there was a significant increase in small intestinal permeability early in life and before the onset of colitis.

When small intestinal permeability was selectively decreased with AT-1001 (a ZOT antagonist peptide) colitis was significantly ameliorated. In contrast, when it was increased with AT-1002 (a ZOT agonist peptide) colitis worsened, indicating that modifications in the paracellular traffic of the small intestine had a significant effect on the severity of colonic disease.

In order to study the possible mechanisms by which small intestinal permeability modulated disease in the colon, we measured the effect of increasing small intestinal permeability on the colonic microbial flora of IL-$10^{-/-}$ mice. After AT-

1002 treatment from 4-12 weeks of age, there was an evident shift in colonic adherent flora. This effect was not a consequence of inflammation as there was a similar effect in wild type mice.

We also studied the effect of increasing small intestinal permeability in the development of oral tolerance to dietary antigens. When wild-type mice were fed OVA under conditions of increased small intestinal permeability there was a significant increase in the proliferation of B cells in the spleen and an increase in OVA-specific humoral response, compared to animals fed OVA alone. Moreover, the production of IL-10 in response to oral OVA was prevented when OVA was given with AT-1002, both in the small intestine and the colon.

The studies presented in the doctoral thesis suggest that small intestinal permeability has a critical role in the development of colitis in IL-$10^{-/-}$ mice, and that increasing paracellular traffic in the small intestine may lead to changes in colonic bacterial flora and the abrogation of tolerance to oral antigens, two features of inflammatory bowel disease in humans.

Preface

This thesis consists of one review paper, one accepted research paper, one book chapter and additional experimental data obtained during the course of my degree. This thesis has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research of the University of Alberta.

Chapter 1 consists of a review paper published in *Gut* (2006; 55:1512-20) and it is coauthored with Dr. Jon Meddings and Dr. Lana Bistritz. My contribution was compiling all relevant information regarding the clinical relevance of intestinal permeability, as well as editing the entire text and some of the figures.

Chapters 2 and 3 include excerpts from a research paper published in *Gut* (2009; 58:41-48) and it is coauthored with Drs. Jon Meddings, Karen Madsen and Jason Doyle. My contribution was in the experimental design, performing all laboratory techniques except histology (performed by Dr. Doyle), data analysis and interpretation. I also wrote the entire manuscript and helped with the editing of it.

Chapters 4 and 5 include excerpts from a research paper submitted for publication to *Gastroenterology,* which is currently under revision. My contribution to this paper included experimental design, all laboratory experiments, data analysis and interpretation. I also wrote the entire manuscript.

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I would like to thank my supervisors Jon Meddings and Karen Madsen for my amazing experience as a doctoral student. From the beginning you supported me in every possible way and I owe my success as a graduate student to both of you. Karen, having you as my supervisor has been priceless. You have been my immediate source for answers and feedback all of these years. Your ability to simplify ideas and to communicate them allowed me to not feel intimidated by the work that needed to be done. I deeply thank you for the trust that you have put in me throughout these years. Above all, I thank you for the example that you have given me as a woman in research. In my experience it is very hard to see such an accomplished woman who manages to juggle an increasingly successful career with life as a wife and as a mother. By doing all of what you do and remaining a sane, happy and approachable person, you have showed me that it is not only possible but that one can excel at it. You have set the bar very high for me but I know now that it is an attainable goal and I thank you for that.

Dr. Meddings, working as your technician for two years and as your student for almost four years has been my most fulfilling academic experience. Learning from you has been an honour; it provided me not just with knowledge but also with the drive to pursue my goals. I deeply thank you for accepting me as your student and providing me with excellent opportunities to complete my doctoral degree, including: your confidence in my abilities to create my own ideas and experiments, and to present them in the numerous meetings that you have allowed and financed me to attend. Your constant positive feedback provided me with the

self-confidence to trust my work, even when things were not going smoothly. Your tactful constructive criticisms encouraged me to learn more and improve my skills. You are truly a model to follow, not just as a professional but also as a human being.

The work presented here in this thesis (as well as the work that is not included here) would have not been possible without the help and feedback of many people in the lab. With you I also share many years of good memories and friendship. Thank you Julia Ewaschuk, Jody Campeau, Sarah MacFarlane, KyungRan Park, Francis Cheung, Naomi Hotte, Matt Emberg, John Walker, Eric Albert, Beate Sydora and Rae Foshaug.

The objectives of my doctoral thesis took me to unfamiliar areas of research and gave me the opportunity to approach other faculty members for help. First, I want to thank Dr. Catherine Field for all of your valuable input during our committee meetings and for your help with many experiments and results. I also thank you for the opportunity to write a book chapter with you and for encouraging me to learn outside of my field. I also give thanks to Dr. Richard Fedorak, Dr. Tom Churchill, Dr. Eytan Wyne and Dr. Colin Anderson, as well as the numerous faculty members that helped me with my doctoral research.

In a personal level, all of these years were a memorable and satisfying experience thanks to the support from my family, both in Canada and in Costa Rica.

Pirru, vos has sido mi fuente de inspiración y de admiración desde hace mucho tiempo. Todo lo que hago y he hecho, incluyendo esta tésis, es un reflejo del ejemplo que me das a diario. Junto a vos siento que puedo hacer cualquier cosa y

sos la persona que mas apoyo me ha dado y quien mas confía en mi. Hacés de mi vida una experiencia tan linda y tan valiosa que si tuviera que elegir una sola cosa en la vida, sería seguir pasando cada día con vos. El agradecimiento que siento con vos es tan increíble que trasciende cualquier palabra que pueda escribir acá. Venirte para Canadá a estar conmigo, apoyarme en cada decisión que he tomado, crecer y superarte como persona de la manera que lo has hecho, pero sobre todo, quererme como me querés, me hace sentirme la persona mas dichosa. Espero que en todo el tiempo que tenemos juntos logre demostrarte lo que significás para mi. Te amo mas que todo y me haces la mujer y muy pronto la mamá mas dichosa de este mundo. A nuestro Pulgarcit@ también le agradezco haber sido la fuente de inspiración escribiendo esta tesis. Tenerte adentro de mi durante estos meses ha hecho de este proceso algo que nunca olvidaré. Cada patadita mientras escribía algún capitulo o trabajaba en alguna figura me recordó que nunca estoy sola y que el equipo Acuña-Arrieta tiene un miembro más. Espero que cuando puedas leer estas palabras sepás que desde ya te amo de una forma sobrenatural que jamás me imaginé y que cuento los días para conocerte.

A mi familia le agradezco muchísimo el apoyo que a diario me dan. A mis papás, desde pequeña me enseñaron a que siempre hay que tener metas y cumplirlas y cada uno de mis logros los debo a Uds. Mamá, nunca me has dejado y te agradezco cada una de las veces que me lo recuerdas. Tu ejemplo como mujer es algo tan inmenso que espero algún dia poder ser la mitad de la persona que fuiste. A mis hermanos les agradezco muchísimo el apoyo que me dan. Uds. me recuerdan el valor de la familia, que pase lo que pase nos tenemos uno al otro y

que a pesar de la distancia siempre estaremos para ayudarnos. A Merce, te agradezco mucho lo que nos quieres a todos en mi familia. Sos un ejemplo de una buena amiga. Los quiero muchísimo.

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List of Abbreviations

ZOT: Zonula occludens toxin

PLC: Phospholipase C

PPI: Phosphatidyl inositol

PPI-3: Inositol 1,4,5-trisphosphate

DAG: Diacylglycerol

PKC-α: Protein kinase-alpha

Pre-HP2: pre-haptoglobin-2

HP: Haptoglobin

DH: Dermatitis herpetiformis

MYD88: Myeloid differentiation primary response gene

TLR: Toll-like receptor

CD: Crohn's disease

NOD2/CARD15: Nucleotide-binding oligomerization domain 2/caspase recruitment domain family member 15

ATG16L: Autophagy related gene 16L

IL: Interleukin

UC: Ulcerative colitis

SAMP: Senescence-accelerated prone

$Mdra^{-/-}$: Mouse downregulated in adenoma

$IL\text{-}10^{-/-}$: Interleukin 10 gene deficient

IBD: Inflammatory bowel disease

Th-1: T helper 1

TNF-α: Tumour necrosis factor-alpha

TGF-β: Transforming growth factor-beta

NK: Natural killer

HLA: Human leukocyte antigen

TCR: T cell receptor

Ig: Immunoglobulin

IFN-γ: Interferon-gamma

SPF: Specific pathogen free

NSAID: Non-steroideal anti-inflammatory drug

HPLC: High performance liquid chromatography

PD: Potential difference

Isc: Short circuit current

PBS: Phosphate buffered saline

ELISA: Enzyme-linked immunosorbent assay

SEM: Standard error of the mean

MAP: Mitogen activated protein

BSA: Bovine serum albumin

MPO: Myeloperoxidase

sIgA: Secretory IgA

MLCK: Myosin light chain kinase

GI: Gastrointestinal

PSA: Surface polysaccharide

PCR: Polymerase chain reaction

TRFLP: Terminal restriction fragment length polymorphism

GALT: Gut associated lymphoid tissue

PP: Peyer's patches

MLN: Mesenteric lymph node

GC: Germinal center

DC: Dendritic cell

PC: Plasma cell

APC: Antigen presentic cell

IEC: Intestinal epithelial cell

OVA: Ovalbumin

mAbs: Monoclonal antibodies

Chapter 1

General Introduction[1]

The goal of this introduction is to describe the barrier function of the intestine, the structure of the tight junction, methods to evaluate intestinal permeability, and most importantly the relevance of abnormal permeability to disease. Finally, I state a series of objectives and specific questions that provide the structure of this doctoral thesis.

1.1 Intestinal barrier function:

From the lower esophageal sphincter to the anus, the gastrointestinal tract has a single contiguous layer of cells that separates the inside of the body from the external environment. Separation is important as there are a wide variety of environmental agents in the lumen of the bowel that can initiate or perpetuate mucosal inflammation if they cross the epithelial barrier. While the epithelial lining of the intestine plays a critical role in preventing access of these agents, it is not the only component of what is termed barrier function. Also important are secreted products such as immunoglobulins, mucous, defensins, and other antimicrobial products.

The importance of epithelial barrier function in normal homeostasis can be appreciated from experiments performed in the early 1990s where cell wall extracts from luminal bacteria were injected into the colonic wall of rats (1). This

[1] *A version of this chapter has been published as a review paper. Arrieta et al. 2006. Gut. 55(10) 1512-20.*

simple manoeuvre of bypassing the epithelial barrier and placing luminal compounds directly into the colonic wall initiated an inflammatory disease that was characterised by granulomatous reactions both in the bowel and, importantly, as a systemic inflammatory process. Similar results were also observed by variably expressing a dominant negative N-cadherin mutant in epithelial cells along the crypt-villus axis. This induced abnormal function of intercellular junctions and initiated a mucosal inflammatory disorder that resembled Crohn's disease (2). The important concept that emerges from these experiments is that by simply abrogating epithelial barrier function, inflammatory disease can be induced in a susceptible host with features that are expressed both locally and systemically.

1.2 Anatomical structure of the epithelial barrier:

Much of the epithelial barrier is formed by the rigid lipid bilayer of the enterocyte brush border. As in most cell membranes, this structure has appreciable solubility to lipid compounds but offers a strong barrier to water soluble constituents. The enterocyte balances its dual function as both an absorptive and barrier cell by embedding transport systems within this membrane for the water soluble compounds that it wishes to transport. However, at the junction between epithelial cells there is a potential route for solute traffic that is not regulated by brush border membrane transporters or channels. In order to regulate traffic through this paracellular pathway, mammalian epithelial cells form a series of intercellular

junctions along their lateral margins. Closest to the luminal surface lies the tight junction and underneath is the adherens junction.

These structures are enormously complex in both their lipid and protein constituents. Ever expanding families of proteins are found in the vicinity of these junctions, forming fibrils that cross the plasma membrane to interact with proteins from the adjoining cell. These proteins also interact intracellularly with the actomyosin ring that encircles the enterocyte at the level of the tight junction through numerous smaller proteins. The fibrils between cells consist of at least two types of tetraspanning membrane proteins, occludin and members of the claudin family. The latter is comprised of at least 19 different but related proteins, their name coming from the Latin "to close". It is of interest that defects in claudins have already been associated with human disease: a genetic mutation in claudin 16 appears to be involved in renal hypomagnesaemia, characterised by massive renal loss of magnesium (3). Furthermore, claudins 3 and 4 are targets for the bacterially produced toxin, *Clostridium perfringens* enterotoxin which dramatically increases tight junctional permeability in tissue culture systems (4).

On the intracellular side of the membrane, the carboxy terminal end of these proteins interacts with the tight junction proteins ZO-1, ZO-2, and ZO-3 (Figure 1.1). These proteins belong to the membrane associated guanylate kinase superfamily and possess an enzymatically inactive guanylate kinase-like domain. Underneath the junctional complex lies a ring of actin microfilaments and contraction of this has been proposed to regulate paracellular permeability.

Connecting this ring to the junctional complex (ZO family members) are a series of actin filaments, as schematically outlined in figure 1.1. In addition to these protein constituents, there are numerous other junctional proteins that have been described and a tremendous effort is underway to elucidate the physiological interactions of these proteins in terms of cellular and junctional function. One emerging concept is that the relative abundance of the different claudin family members is important in determining the physiological properties of the junction. Along normal developmental axes (such as along the crypt-villus axis) or during disease expression, the relative abundance of the claudins can change by up to 1000-fold while other more structural protein components seem to change relatively little (5).

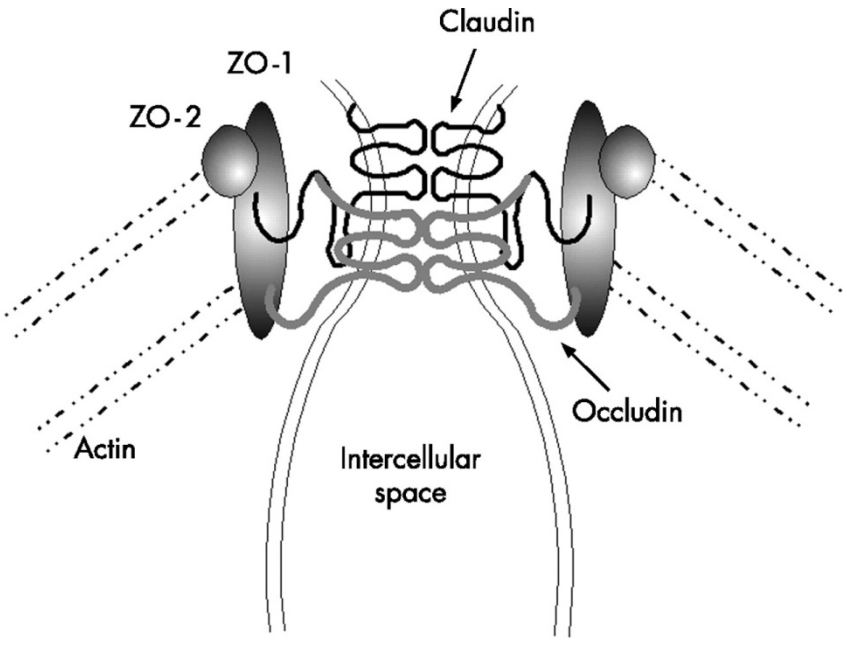

Figure 1.1. Tight junction structure. Homotypic association of claudin and occludin is illustrated.

It is also important to consider what factors alter permeability of the junction. It is now recognised that the functional state of the tight junction, once considered a static parameter, is in reality incredibly dynamic. Epithelial tight junctions open and close all the time in response to a variety of stimuli. These include dietary state, humoral or neuronal signals, inflammatory mediators, mast cell products, and a variety of cellular pathways that can be usurped by microbial or viral pathogens (6-15). A complete discussion of all of these mechanisms is beyond the scope of this thesis but a few deserve closer attention as they are important in the understanding of disease pathogenesis.

The first are dietary factors. It is now generally appreciated that the permeability of the paracellular pathway can be modulated by transcellular absorptive processes. During activation of the sodium dependent glucose transporter (SGLT1), there is a physiological opening of tight junctions that allows for the movement of small molecules and peptides(16-19). This pathway will accommodate particulate sizes of the order of 2000 molecular weight (MW) but still exclude large particles such as horseradish peroxidase (MW ~40 000)(20). Although this is a normal physiological event, the purpose it subserves remains unclear. However, as discussed in the next section, this observation is important in the understanding of how to understand permeability measurements.

Other relevant pathways are the ones used by bacteria to enter the host. Many bacteria alter the tight junction, presumably to enhance their own growth requirements. *Vibrio cholerae* secretes a variety of toxins and one of these, zonula

occludens toxin (ZOT), was recognised as increasing paracellular permeability. The mechanism by which this occurred was novel and involved binding to an apical membrane receptor on the enterocyte with subsequent activation of an intracellular pathway resulting in actomyosin contraction and increased paracellular permeability (Fig. 1.2).

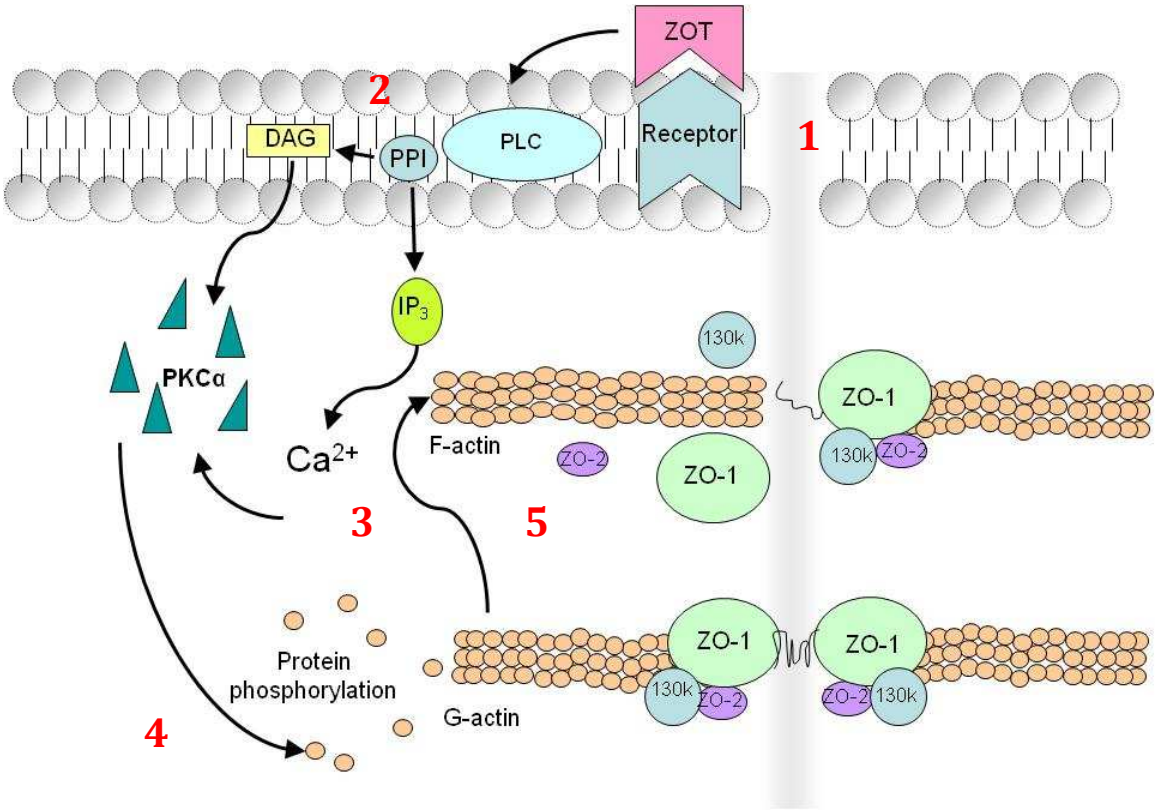

Figure 1.2 The ZOT pathway. 1. ZOT binds to its apical receptor which activates phospholipase C (PLC). 2. PLC cleaves phosphatidyl inositol (PPI) into inositol 1,4,5-tris phosphate (PPI-3) and diacylglycerol (DAG). 3. Activation of protein kinase C-alpha (PKCα) occurs directly (via DAG) or through the release of intracellular Ca^{2+}. 4. PKCα phosphorylates proteins of the actin cytoskeleton, changing its conformation from G-actin to F-actin. This change in conformation

mechanically shifts the cytoskeleton and causes the displacement of the proteins of the tight junction, loosening it. Modified from (6).

The same investigators speculated that it was unlikely that this pathway was present for the sole benefit of the bacteria and that a more likely scenario was that the pathway was a physiological one that bacteria had evolved to take advantage of. This proved to be true and in an elegant piece of work antibodies specific to this bacterial toxin were used to identify the human homologue for this pathway now termed zonulin (21). It appears that in many scenarios where permeability is increased, a common pathophysiological event is upregulation of zonulin secretion from a lamina propria source into the lumen with inappropriate activation of this pathway, although the source has not been identified yet (22-24). The end result is increased paracellular permeability. More recently however, the same authors that discovered zonulin elucidated its proteomic sequence and it does not appear to have homology with ZOT. Zonulin has been identified as the precursor for haptoglobin-2 (pre-HP2), the uncleaved form of haptoglobin (HP), a protein that scavenges free hemoglobin to prevent its oxidative activity (25), and has also been shown to act as a monocyte chemoattractant (26).

1.3 Measurement of intestinal permeability:

Over the past several decades there has been a concerted effort to develop simple non-invasive means to evaluate the permeability properties of the paracellular pathway. In order to rationally evaluate methods to quantitatively evaluate the paracellular pathway *in vivo*, it is important to keep in mind a few simple principles. Movement across this pathway occurs by a non-carrier mediated process and as such depends on several features.

- The concentration gradient across the barrier.
- The surface area of the epithelium.
- The time available for permeation.
- The intrinsic permeability properties of the barrier.

As the last item is the characteristic of interest for measurement, it is important to try and avoid differences in the first three variables during the measurement process by carefully selecting the probes used and the method of their employment. Historically, a wide variety of probes have been utilised to determine paracellular permeability properties. Typically, they have several features in common; they are usually small, water soluble, non-charged compounds that are not destroyed in the gut, are non-toxic, not metabolised or sequestered once absorbed, and quantitatively cleared by the kidney into the urine. Finally, they should be easily detectable in urine and easily separated from similar endogenous or dietary compounds.

Despite the proliferation of available probes, the majority of work in humans and experimental animals has employed a variety of small saccharide probes and/or Cr-EDTA. This is because these probes satisfy the criteria above, are inexpensive, and easily detectable. However, it is important to recognise that several of these probes are destroyed by processes that take place in the lumen of the gut and this limits their exposure to the epithelium in a manner that can be advantageously used to evaluate permeability characteristics in a regional manner.

As an example, sucrose is a useful probe for determining permeability characteristics of the gastroduodenal region (27). Distal to the gastroduodenal region, sucrose is rapidly hydrolysed by sucrase-isomaltase and therefore permeation of intact sucrose across the gastrointestinal mucosa must occur in the most proximal regions of the gut. In a similar manner, the traditional small intestinal permeability probes, lactulose, mannitol, rhamnose, or cellobiose, are degraded by the bacterial flora of the large intestine and yield no information regarding colonic permeability characteristics. Furthermore, under conditions of small intestinal bacterial overgrowth, degradation of lactulose and mannitol is impossible to quantify and calls into question determination of permeability. In order to evaluate colonic permeability properties, probes must be selected that are stable in this environment. These include either Cr-EDTA or sucralose (28). Although these probes are stable throughout the gastrointestinal tract, they preferentially provide information regarding colonic permeability because, under normal conditions of intestinal transit, they reside within the colon for the majority of their time in the gut. The principles and techniques of probe selection for

regional permeability determinations are discussed more extensively elsewhere (28) but the basic features of these probes are schematically depicted in figure 1.3.

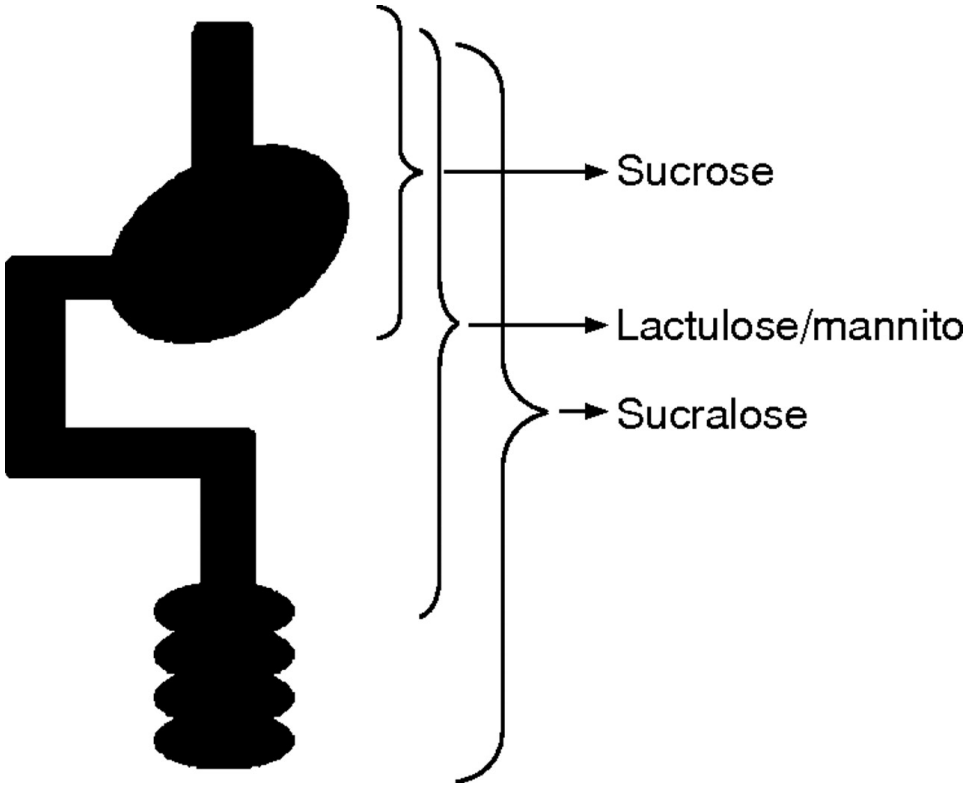

Figure 1.3. Region-specific permeability measurement. By carefully choosing probes that have only a limited exposure to the gastrointestinal epithelium site, selective permeability determinations can be made. Sucrose is destroyed once it leaves the stomach and so sucrose permeability is a reflection of gastroduodenal disease. In a similar manner, lactulose and mannitol are hydrolyzed in the caecum and provide information regarding the small intestinal epithelium. Finally, probes such as sucralose and Cr-EDTA are stable throughout the gut. They provide preferential information regarding the colonic epithelium as they spend most of a 24-hour exposure period in that organ.

Perhaps the most important issue to consider is what the permeation rate of the various probes actually means. The most concise hypothesis regarding the routes various probes take across the epithelium has been provided by Fihn and colleagues (29). These investigators provided data to suggest that there are a series of "aqueous pores" distributed along the crypt-villus axis of the small intestine. At the tips of the villus are relatively abundant small channels (radius <6 A) while in the crypts there exist much larger channels (50–60 A) in low abundance. At the base of the villus are intermediate sized (10–15 A) channels. The channels at the tips of the villus are increased in number by addition of glucose (18) and susceptible to solvent drag effects while those in the crypt are unaffected by these alterations. The intermediate sized channels seem to be unaffected by solvent drag effects, perhaps as under physiological conditions this part of the villus is not exposed to luminal contents. With these data it would appear that, under normal conditions, molecules the size of disaccharides (for example, lactulose) are restricted from moving across the villus tip whereas mannitol can do so with relative freedom.

Traditionally, small intestinal permeability is expressed as the ratio of the fractional excretion of a larger molecule to that of a smaller one (for example, lactulose:mannitol). Using the paradigm from the preceding paragraph, this would represent the number of intermediate sized pathways as a proportion of the total number of aqueous channels. As the smaller channels are concentrated at the villus tips, permeation rates of compounds across this pathway become a rough assessment of mature small intestinal surface area. This is well established from a

clinical perspective. In diseases where there is a marked reduction in mature small intestinal surface area, such as celiac disease, there is a substantial reduction in the fractional excretion of small probes such as mannitol. Coupled with this there is an increase in the fractional excretion rates of larger probes such as lactulose. This would suggest either an increased number of intermediate sized channels per unit surface area or the appearance of an alternate pathway, accessible to lactulose, which is not evident under normal physiological circumstances. As intermediate sized channels appear to be present in the immature parts of the crypt-villus axis, relative expansion of this crypt-villus fraction (as observed in celiac disease) is a possible explanation. However, there are also data demonstrating that the presence of either epithelial damage or increased rates of apoptosis provide alternate routes for the permeation of larger molecules such as lactulose (30-32).

The net result of a reduction in the fractional excretion of mannitol and an increase in that of lactulose is a dramatic increase in the lactulose:mannitol ratio. The converse is seen during the healing process of the celiac lesion. During treatment with a gluten free diet, the first sign of recovery is seen as an increase in the fractional excretion of mannitol, suggesting that recovery of mature surface epithelium precedes the reduction in apoptosis and damage (33). This interpretation is consistent with the known pathophysiology of celiac disease.

Therefore, a reasonable interpretation of permeability data, expressed as a lactulose:mannitol ratio, is the amount of epithelial damage as a proportion of mature small intestinal surface area. It is important to remember that this

parameter provides no information about either colonic or gastric permeability. The former is due to the fact that both probes are destroyed in the colon and the latter since the surface area of the small intestine is so much greater than that of the stomach. In order to obtain this type of information, additional probes such as sucrose and sucralose are necessary and provide the necessary data without detracting from the measurement of small intestinal permeability. Additional probes to assess absorptive function can also be added to extend the usefulness of such testing (34;35).

1.4 Intestinal permeability and disease:

The concepts of intestinal permeability and disease have been related for years. Next is a summary of three important diseases in which there is accumulating evidence of intestinal permeability having an important role in their pathogenesis, along with the presence of a triggering antigen, and a genetic predisposition to react abnormally to this trigger.

1.4.1 Diabetes type 1

Increases in intestinal permeability in type 1 diabetics were first reported 20 years ago (36). More recent studies, that have excluded celiac disease patients, continue to find that type 1 diabetic patients have increased small intestinal permeability (37;38).This is not simply related to hyperglycemia as patients with type 2 diabetes have normal permeability (39).

More interestingly, a study done with 339 type 1 diabetic patients and 89 of their first degree relatives showed that diabetic patients had significantly higher serum zonulin levels than either controls or their relatives. Zonulin is a recently described peptide that opens epithelial tight junctions and increases small intestinal permeability (21). Furthermore, by examining serum of these patients and their relatives for HLA testing, these authors also demonstrated that "pre-diabetics" (positive for IA2 autoantibodies but before the onset of diabetes) had elevated zonulin levels in seven of 10 specimens. Patients with increased zonulin, not unexpectedly, also had increased permeability (39). Probably the best example comes from an animal model, the BB rat. In this model of autoimmune diabetes an inbred line of rat develops classical autoimmune diabetes when weaned onto a normal diet (40). However, if the animal is instead weaned to a hydrolysed diet, a much lower incidence of diabetes results. This is an inbred line of animals and so a genetic susceptibility of the mucosal immune system is not an unreasonable proposition. However, the observation that simply altering the diet can dramatically change the risk of disease suggests a dietary trigger that can be removed by protein hydrolysis. When measured, there is a marked increase in permeability of both the small intestine (lactulose/mannitol ratio) and of the stomach (sucrose) (41). The increase in permeability was not due to diet as animals receiving the hydrolysed diet had the same increase as those on the regular chow diet. The increase in permeability is not present at all ages and so the question arises as to what is the mechanism of the increased permeability. As mentioned previously, one potentially abnormal pathway is the zonulin system to

open the paracellular pathway. Recent work has demonstrated that in the BB rat, coincident with the increase in permeability, there is increased zonulin secretion into the lumen (42). Therefore, in this model of autoimmune disease there is now evidence for a dietary antigen and an abnormality of the zonulin pathway that leads to increased permeability. The disease in this animal model can be prevented by a change in the diet, but most exciting of all, it can also be prevented by abolishing the increased permeability. This was recently demonstrated by Watts *et al* using a zonulin receptor antagonist which prevented the increase in small intestinal permeability and the development of diabetes (42).

Therefore, the data seem fairly convincing that in both animal models and human type 1 diabetes, a substantial percentage of individuals have increased permeability that appears to precede the disease state and may play a role in pathogenesis.

1.4.2 Celiac disease

Celiac disease also appears to follow very similar characteristics. It is an exceedingly common disease that may affect up to 1% of the population and has clear genetic and environmental components. From a genetic perspective, almost all celiac patients (95%) are HLA-DQ2 positive while the remainder carries HLA-DQ8 (43). There is also evidence of other important genetic linkages (44). With this disease we have the good fortune to have identified the inciting environmental antigen—gluten. Removal of this protein prompts complete remission of all

clinical symptoms of the disease, including a return of abnormal intestinal permeability to almost the normal range in the majority of subjects (45). In fact, an increase in permeability is a sensitive test for the presence of even small amounts of gluten in the diet (45). However, if intestinal permeability normalises during disease remission, there is increasing evidence that intestinal permeability plays a role in the etiology of this disease. The first line of evidence comes from animal studies. An inbred Irish Setter dog line develops a gluten sensitive enteropathy that mimics human celiac disease. In these animals the disease can be completely prevented by weaning the animal onto a gluten free diet. However, subsequent exposure to the antigen immediately prompts the reoccurrence of the clinical symptoms of the disease. Importantly, animals that have never been exposed to dietary gluten have increased small intestinal permeability (46). This strongly suggests that in this animal model abnormal permeability precedes disease.

In humans the data are more difficult to interpret, as there is insufficient data in individuals prior to the onset of disease. However, with careful analysis it is apparent that tight junctional structure is abnormal in children with celiac disease (47;48). In some studies gluten removal does not totally resolve the defect, suggesting that either some portion of the damage is irreversible or that there is a prior genetically-induced alteration in the tight junction (48). From a functional viewpoint, intestinal permeability has not been well assessed in relatives of patients with celiac disease. In one study about one third of first degree relatives had abnormal permeability (49). Approximately 8% of these had a positive

endomysial antibody test, underwent biopsy, and were demonstrated to have asymptomatic celiac disease but the reason for the abnormal permeability in the remainder was not reported.

Patients with dermatitis herpetiformis (DH) provide an interesting perspective in this regard. Subjects with this condition exhibit an enormous range of associated bowel pathology from frank celiac disease to a completely normal intestinal biopsy and no evidence of bowel disease. In a recent study of 18 such patients (24) it was noted that all DH patients had increased intestinal permeability, including those patients without evidence of intestinal disease. Furthermore, these patients also had elevated serum zonulin levels, suggesting that the abnormal permeability was associated with, and perhaps caused by, an abnormality of the zonulin pathway. As some of these patients may go on to develop celiac disease, it would appear that in these cases increased permeability would precede development of disease and a potential mechanism of this is upregulation of the zonulin pathway.

The association of abnormal permeability and increased zonulin secretion in a gluten sensitive disease (DH) is intriguing. Recent work suggests that in celiac disease, gluten functions as a trigger for zonulin release which subsequently increases paracellular permeability (50). These events were found to be dependent on the myeloid differentiation primary response gene (MyD88) suggesting the involvement of a Toll-like receptor (TLR) pathway that did not appear to be either TLR2 or TLR4. The authors suggest a model of celiac disease in which gluten

first interacts with the innate immune system through a TLR pathway. This subsequently induces release of zonulin from a lamina propria source, increased paracellular movement of gluten, and enhanced interaction of gluten with the mucosal immune system initiating the inflammatory disease process.

1.4.3 Crohn's disease (CD)

This disease is significantly attenuated or does not develop in susceptible animal models under germ free conditions (51) or in humans where luminal contents are diverted. From these data it would appear that luminal antigens or other factors are critical. A genetic component is also apparent and several gene mutations have been identified, including those in nucleotide-binding oligomerization domain 2 or caspase recruitment domain family member 15 (NOD2/CARD15) (52) as well as in the organic cation transporter (OCTN or IBD5) (53;54). More recent genetic associations involve the autophagy related gene ATG16L (55) and the interleukin-23 receptor (IL-23R) gene (56). Not surprisingly, many of these genes are involved in immune functions and barrier function.

In the human condition it is also evident that increased small intestinal permeability is commonly observed in populations at high risk of developing CD (57-62). Increased permeability is observed in the absence of symptoms of disease, suggesting that it is not merely an early manifestation of CD. There is now one case report of a young woman found to have abnormal permeability during a family study who subsequently developed CD (63). At the time when her permeability was increased, extensive investigations revealed no evidence of

bowel disease. It took years for CD to develop. There are also data to suggest that patients with increased permeability are also likely to have evidence of subclinical inflammation, as assessed by calprotectin excretion (64).

There is also an apparent link between increased permeability and the mutation in the NOD2/CARD15 gene. In a recent study, Buhner *et al* demonstrated that not only is the risk of having abnormal permeability and a NOD2 mutation increased among first degree relatives of those with CD, but these are related. Relatives with a NOD2 mutation had a 75% chance of having increased permeability (65), suggesting that this mutation may influence epithelial permeability. The mechanism by which this might occur is unclear. The NOD2 gene product is present in enterocytes and in Paneth cells. In enterocytes this mutation induced an elevated activation of the nuclear factor kappaB (NF-κB), a key transcription factor in the response to bacterial antigens (66). In Paneth cells the mutation is associated with a reduction in secretion of defensins (67), an observation previously reported to be associated with CD (68). This may represent an important link as reduced defensin secretion would be expected to alter the luminal microflora which in turn can have profound effects on epithelial permeability by many different mechanisms (69-71).

While an altered microflora, either induced by altered Paneth cell secretion or other mechanism, can directly increase permeability, there are other potential etiologies. Altered expression of tight junction proteins may also increase paracellular permeability. In fact, increased expression of claudin-2 has been

demonstrated to increase permeability (72), presumably as homodimers of this protein form associations that are not as "tight" as heterodimers with other claudins. In this regard, it is of interest to note that upregulation of claudin-2 expression has been reported in patients with CD and ulcerative colitis (UC) (73). Whether this is an effect of the inflammation itself or a pre-existing abnormality could not be determined. However, other authors have reported that the abnormality in expression of junctional proteins is specific to biopsies taken from inflamed regions of UC and CD patients; those portions of the gut that were not inflamed appeared to be similar to the control biopsies in terms of junctional proteins (74).

In terms of function, Soderholm and colleagues (75) reported that baseline permeability in non-inflamed portions of gut taken from patients with CD is similar to controls, but following exposure of the tissues to a mildly damaging agent (sodium caprate) the non-inflamed tissue demonstrated a marked increase in paracellular permeability. This would suggest that there is a subtle alteration of function, independent of inflammation, which can be manifest as an increase in paracellular permeability. Similar findings are observed *in vivo* manifesting as hyperresponsiveness of the small intestine to the damaging effects of non-steroidal anti-inflammatory compounds such as aspirin or ibuprofen (76;77).

In animal models of CD, the senescence-accelerated-prone (SAMP) mouse, and the mouse downregulated in adenoma ($mdra^{-/-}$), it has now been convincingly

demonstrated that abnormal permeability is present prior to expression of the inflammatory disease (78-80).

Another animal model of CD, the IL-10 gene deficient (IL-10 $^{-/-}$) mouse, is predisposed to a proinflammatory state because it lacks IL-10, an anti-inflammatory cytokine. This mouse, which is the model used in *in vivo* experiments in this thesis, develops a patchy, chronic colitis similar to human CD (81). From the point of view of this study, the IL-10 $^{-/-}$ mouse model of disease has two important features. Firstly, these animals have been described as having increased small intestinal permeability that has been observed very early in life and well before the onset of disease (82). Secondly, disease development is dependent upon luminal factors: it does not occur in animals raised under germ free conditions. These observations suggested that the colitis observed in these animals might develop as a consequence of abnormal small intestinal permeability with increased presentation of a luminal agent to a genetically predisposed mucosal immune system.

Taken together, these data support the hypothesis that there is a permeability abnormality in CD and that this abnormality could be important in the genesis of disease. However, it is still unclear why this defect is present in the first place and more importantly, if this defect can cause disease, or if it is disease that causes the gut to leak. The purpose of my thesis is to establish this cause-effect relationship.

HYPOTHESIS

Increased in intestinal permeability precedes and causes colitis in the IL-10$^{-/-}$ mouse model.

1.5 Objectives

The following are the objectives and research questions initially proposed to test this hypothesis:

A. To determine the site of the gut permeability defect present in the IL-10 $^{-/-}$ mouse and determine if this is an inherent defect or a product of inflammation.

A.1 Does the IL-10 $^{-/-}$ mouse have a defect in stomach, small and/or large intestinal permeability?

A.2 If so, Is this defect present early in life?

A.3 Does the defect in gut permeability precede signs of disease?

B. To establish if small intestinal permeability is a requirement for colitis in the IL-10 $^{-/-}$ mouse.

B.1 Can disease be abolished or attenuated if small intestinal permeability is decreased?

B.2 Can disease be exacerbated if small intestinal permeability is worsened?

C. To explore some of the mechanisms by which increased small intestinal permeability may lead to colitis in the IL-10 $^{-/-}$ mouse.

C.1 Can an increase in intestinal permeability induce a change in colonic microbial flora?

C.2 Can an increase in intestinal permeability lead to a change in the mucosal immune cells, inducing loss of tolerance to an antigen presented paracellularly?

Each one of these objectives and specific questions will be discusses in the next four chapters.

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Chapter 2

Intestinal permeability in the IL-10 $^{-/-}$ mouse. [2]

2.1. Introduction

The IL-10$^{-/-}$ mouse, generated in 1993 by Kuhn et al (1), develops a spontaneous and progressive enterocolitis similar to CD and has been widely used as an animal model of inflammatory bowel disease (IBD). The generation of this mouse model emphasized the already established importance of IL-10 in immune homeostasis and intestinal health. This cytokine is key in inhibiting T-helper (Th)1-type immunity by downregulating the production of IL-12 and tumour necrosis factor-alpha (TNF-α), and inducing the production of transforming growth factor beta (TGF-β), a cytokine also involved in Th1-type immune regulation. IL-10 is also a stimulant of B-cell response and antibody production, and it inhibits macrophages, natural killer (NK) cells and Th1-type T cells *in vitro* (2).
Interestingly, more recently developed animals models with deficiencies in molecules involved in the IL-10 signaling pathway (CRF2-4 and STAT-3) also develop a similar enterocolitis (3;4).
Other rodent models generated by deleting or modifying genes that encode other important immune molecules, such as IL-2, TGF-β, IL-7, TNF-α, human leukocyte antigen (HLA)-B27, and T cell receptor alpha (TCR-α) also develop intestinal inflammation, suggesting that IBD develops as a multifactorial process

[2] *A version of this chapter has been published as part of a research paper. Arrieta et al. Gut, 2009. 58(1);41-48*

where immune homeostasis is broken by the absence or the over expression or under expression of immune components. (5).

Depending upon the animal facility, disease in the IL-10$^{-/-}$ mouse has been shown to develop as early as 3weeks of age, when mice start developing multifocal infiltrates in the lamina propria of the cecum, and ascending and transverse colon (6). IL-10$^{-/-}$ mice also develop anemia, leukocytosis, splenomegaly, elevated intestinal permeability and segmental inflammation mainly in the colon, with occasional transmural ulcers (1;6;7). Histologically, these mice develop multifocal lesions throughout the large intestine, characterized by epithelial hyperplasia, and crypt branching. The lamina propria is heavily infiltrated with diverse immune cells, including neutrophils, macrophages, giant multinucleated cells, Immunoglobulin(Ig)A positive B cells and activated T cells. By 6 months of age, the lesions become aggravated and may evolve into adenocarcinomas. The intestinal cytokine profile in these animals is characterized by elevated concentrations of IL-1, IL-6, interferon gamma (IFN-γ), TNF-α, as well as high nitric oxide levels (6). Cell transfer experiments determined that the inflammation in these animals is dependent on IL-10$^{-/-}$ CD4+ T cells and not B cells (8).

The severity of disease in the IL-10$^{-/-}$ mouse is dependent upon the genetic background and the environment in which the mouse is raised (1;6). When raised under conventional conditions IL-10$^{-/-}$ mice develop the most severe lesions, involving the colon and occasionally the small intestine. Disease manifests less severely and is restricted to the colon when animals are raised under specific pathogen free (SPF) conditions (1;6). In contrast, mice raised in germ-free

conditions do not develop disease (9). Furthermore, reconstitution studies with specific microbial strains have shown that different bacterial species lead to varying degrees in disease severity. For instance, axenic mice reconstituted with *Pseudomonas fluorescens, Helicobacter hepaticus* or *Bacteroides vulgatus* remain disease free, where as *Escherichia coli*-associated mice develop mild colitis. In contrast, germ-free animals mono-associated with *Enterococcus faecalis* or *Enterococcus cloacae* develop a more severe colitis (10-13). It is unclear how certain microorganisms are able to induce the immune changes that lead to colitis in this mouse model and how others do not. One possibility is that some bacteria are able to disrupt the intestinal epithelial barrier and come in contact with the underlying lamina propria immune cells, where they begin an immune reaction that in the absence of IL-10 becomes chronic. An interesting study by Sydora *et al* (13) supports this hypothesis by demonstrating that although *B. vulgatus* does not induce colitis in IL-$10^{-/-}$ animals, it did induce disease when the mice were previously treated with indomethacin, a non-steroideal anti-inflammatory drug (NSAID) that disrupts epithelial permeability.

When measured in Ussing chambers, the intestinal permeability of the IL-$10^{-/-}$ mice was elevated as early as 2 weeks of age, both in the colon and the ileum, but permeability appeared normal in the intestines of axenic IL-$10^{-/-}$ mice, suggesting that the microbial flora is what drives a disruption in the epithelial barrier and the subsequent chronic inflammatory response (7). In order to further characterize the intestinal permeability in this animal model and to study its association with the disease process, we measured permeability weekly and *in vivo* in different

sections of the gut from 4-17 weeks of age and compared it to the intestinal permeability of wild type animals. We also measured the secretion of proinflammatory cytokines at different time points and determined a relationship between the permeability defect and the appearance of inflammation in the gut of the IL-10$^{-/-}$ mice.

2.2 Materials and Methods

2.2.1 Animals

Homozygous IL-10$^{-/-}$ mice generated on a 129 Sv/Ev genetic background, and 129 Sv/Ev controls were housed under SPF conditions until weaning (3 weeks), when they were moved to conventional animal housing. The mice were housed in HEPA filter cages and fed a standard mouse chow diet. These mice were bred and raised in the animal facility at the University of Alberta. All animal procedures were conducted in accordance with protocols approved by institutional Animal Care and Use Committees.

Three and a half week old IL-10 $^{-/-}$mice and 129 Sv/Ev controls were randomized into two groups (n=10-13 for each group).

2.2.2 In-vivo permeability measurement

Once every week, all mice were housed in metabolic cages after a 4 hour fast of food and water and immediately after a gavage of 0.2 ml of a sugar probe containing 100 mg of sucrose, 12 mg of lactulose, 8 mg of mannitol and 6 mg of

sucralose. After a 22-hour collection of urine the animals were placed in their respective cages, and provided with food and water.

Urine from each animal was collected in tubes treated with 100 µL of a 10 % Thymol solution (1.0 g/ 10 mL isopropanol) and 100 µL paraffin oil (to prevent urine evaporation). Samples were frozen at -70ºC until analysis. All sugars were quantified by ion exchange high performance liquid chromatography (HPLC) as previously described (14). Briefly, cellobiose was added as an internal standard, and the urine was filtered through a 0.4-µm filter and diluted as necessary. Samples were deionized and then injected on a Dionex MA-1 ion exchange column. Sugars were eluted with NaOH at a flow rate of 0.4 ml/min. Peaks were detected using pulsed amperometric detection on a Dionex HPLC and quantitated as peak areas. Final data were reported as either fractional excretions (sucrose and sucralose) or as a ratio of fractional excretions (lactulose-mannitol). Fractional excretion is defined as the fraction of the gavaged dose recovered in the urine sample.

Sucralose was also assayed by HPLC. Separation was achieved using a Dionex Ionpac NS1 column and acetonitrile-water as the eluent at a flow rate of 1 ml/min. Detection was performed with an electrochemical detector in a fashion identical to the other sugars. For these assays, the internal standard used was phenyl-β-D-thiogalactoside (Sigma Chemical) added to the initial urine sample at a concentration of 0.1 mg/ml.

2.2.3 Ussing chamber assay

Four animals from each group were used for the study of epithelial transport function at 8 weeks of age. Mice were anesthetised and then euthanized by cervical dislocation and a segment of proximal jejunum and proximal colon removed. The same sites were used in all mice. The mucosa was mounted in Lucite chambers exposing mucosal and serosal surfaces to 10 ml of oxygenated Krebs buffer (in mM: 115 NaCl, 8 KCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 2 KH_2PO_4, 225 $NaHCO_3$; pH 7.35). The buffers were maintained at 37°C by a heated water jacket and circulated by CO_2/O_2. Fructose (10 mM) was added to the serosal and mucosa sides. For measurement of basal mannitol fluxes, 1 mM of mannitol with 10µ:Ci [H^3] was added to the mucosal side. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (Isc) with an automatic voltage clamp (DVC 1000 World Precision Instruments, Sarasota, FL), except for 5-10 s every 5 minutes when PD was measured by removing the voltage clamp. Tissue ion resistance (1/G) was calculated from PD and Isc according to Ohm's Law (15). Baseline Isc and conductance (G) were measured after a 20-minute equilibration period. Increases in Isc were induced by addition of the adenylate cyclase-activating agent, forskolin (10^{-5} M), to the serosal surface. Epithelial responsiveness was defined as the maximal increase in Isc to occur within 5 minutes of exposure to the secretagogue.

2.2.4 Mucosal Cytokine Secretion

At 4, 8 and 17 weeks of age, small intestinal and colonic organ cultures were prepared from wild type and IL-$10^{-/-}$ mice. Because of the patchy nature of colitis in IL-$10^{-/-}$ mice whole colons and ileums were removed, flushed with phosphate buffered saline (PBS), and ¼ of the tissue was cut longitudinally and resuspended in tissue culture plates (Falcon 3046; Becton Dickinson Labware, Lincoln Park, NJ) in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml). Cultures were incubated at 37°C in 5% CO_2. After 24 h, supernatants were harvested and stored at −70°C for analysis of cytokine levels. Pellets were left to dry for 4 days and weighed. TNF-α and IFN-γ levels in cell supernatants were measured in triplicate using enzyme-linked immunosorbent assay (ELISA) kits (Medicorp, Montreal, Quebec).

2.2.5 Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM). Comparisons between groups were made using Student's t test or ANOVA with a Tukey test for post hoc comparisons. All calculations were performed using Graphpad Prism 3.0 (San Diego, CA), and significance was assumed at $P < 0.05$.

2.3 Results

To determine whether there was a permeability defect in IL-10$^{-/-}$ mice, we examined small intestinal permeability from 4-17 weeks of age and compared it to wild-type mice. The IL-10$^{-/-}$ mice showed an increased small intestinal permeability beginning at 4 weeks on compared to the Sv/Ev 129 wild-type mice. There was no significant difference in gastroduodenal permeability between the two groups whereas colonic permeability was similar between groups during the first weeks but as disease progressed sucralose excretion increased significantly in IL-10$^{-/-}$. (Fig 2.1).

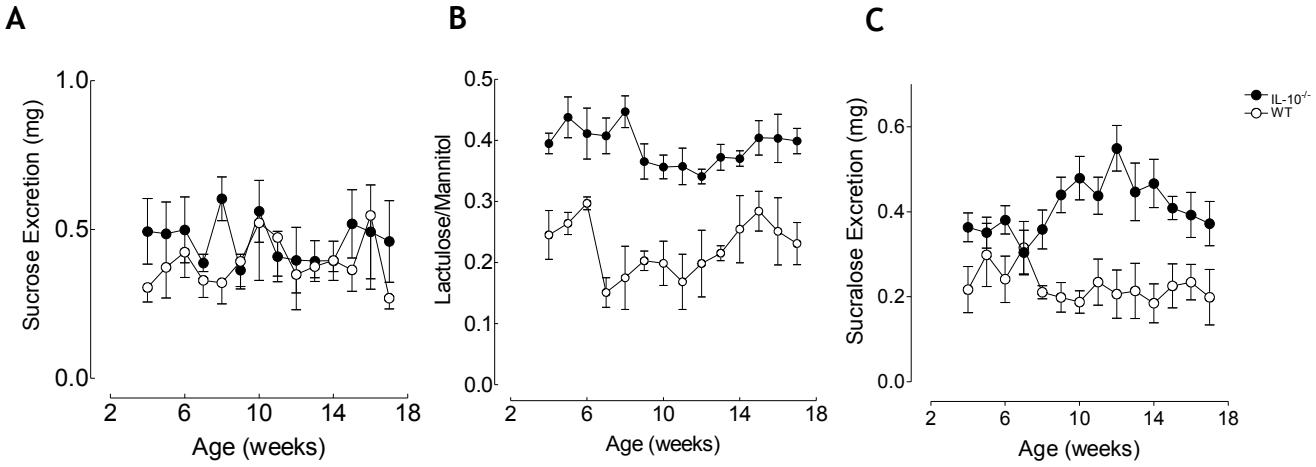

Figure 2.1 Intestinal permeability of IL-10$^{-/-}$ mice. Gastroduodenal (A), small intestinal (B) and colonic (C) permeability measurements of wild type (open circles) and IL-10$^{-/-}$ mice (closed circles) from 4-17 weeks of age. Mice were given a mixture of sugars orally and their urine was collected over 22 hrs. Sugar excretion was measured by ion exchange chromatography (n=10-13).

We also measured small intestinal permeability *ex vivo* at 8 weeks of age in Ussing chambers and there was a significant increase in mannitol flux and a decrease in electrical resistance in the ileums of IL-10$^{-/-}$ mice compared to wild type mice (Fig. 2.2). These results suggest that, using *in vivo* and *ex vivo* methods, the permeability defect in these mice is in the small intestine and that it precedes the onset of disease, which in our IL-10$^{-/-}$ colony occurs after 12 weeks of age. To demonstrate this, we measured the concentration of proinflammatory cytokines IFN-γ and TNF-α in the small intestine and the colon of these mice at 4, 8 and 17 weeks of age. These cytokines have been shown to be elevated in the colon of this animal model in the presence of colitis (16). As shown in Fig 2.3 the IL-10$^{-/-}$ and wild-type mice produced similar levels of IFN-γ and TNF-α in both the small intestine and the colon at 4 and 8 weeks but their cytokine secretion levels were significantly greater at 17 weeks of age, when the animals exhibited colitis. It is important to note that although cytokine levels are significantly elevated in the small intestine, histological damage was not observed in the jejunum or ileum (data not shown). Clearly, abnormal small intestinal permeability preceded the development of colitis by this criterion.

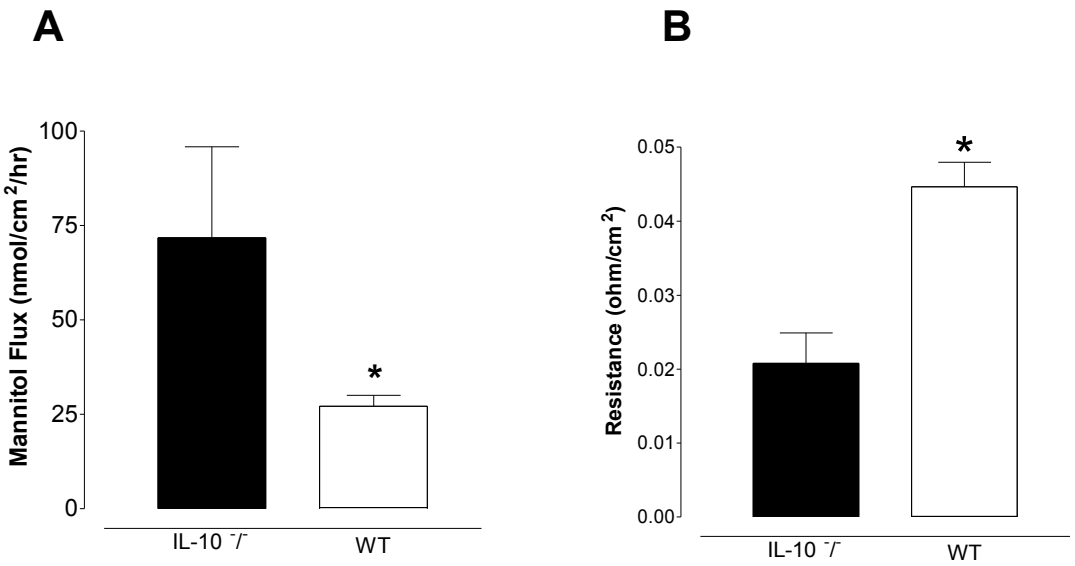

Figure 2.2 Mannitol permeability (A) and electrical resistance (B) across the small intestine was measured in Ussing chambers at 8 weeks of age. IL-10 $^{-/-}$ mice differed significantly from wild-type mice; $p < 0.05$, n=8.

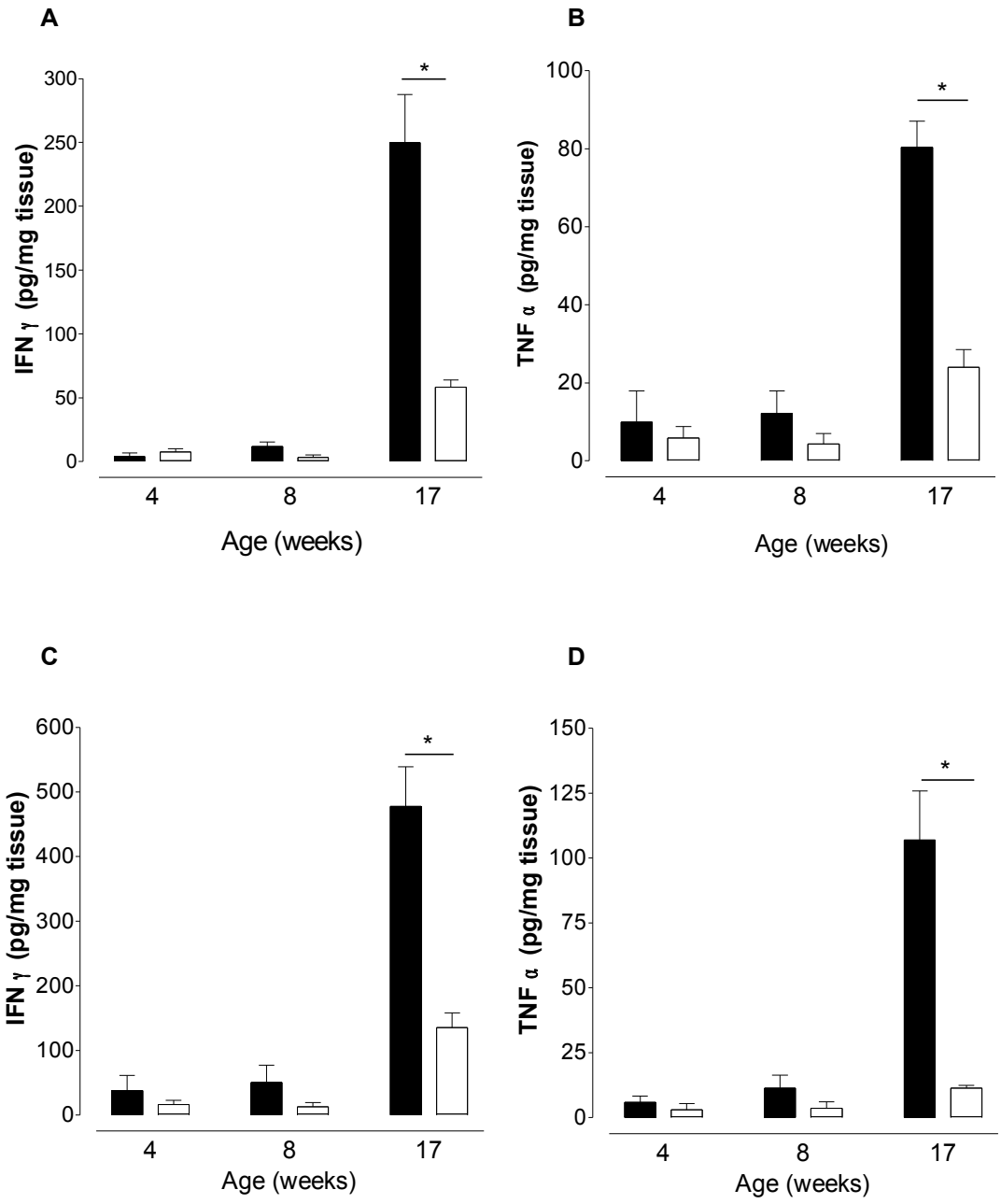

Figure 2.3 Concentration IFN-γ (A and C) and TNF-α (B and D) in small intestines (A and B) and colons (C and D) of IL-10 $^{-/-}$ (black bars) and wild type mice (clear bars) at 4, 8 and 17 weeks of age. For both cytokines and tissues, a significant increase was observed between the IL-10$^{-/-}$ and wild type mice only at 17 weeks, $p < 0.001$, n=4-7.

2.4 Discussion

The IL-10$^{-/-}$ mouse has been widely used to answer important questions regarding the pathogenesis of IBD as it shares similar characteristics with CD in humans. Like human CD, disease in these animals occurs because there is a genetic susceptibility and the presence of an antigenic trigger. Although this trigger has not been elucidated yet, it is widely accepted that it resides in the complex intestinal microbial flora, and that it is probably not a single microorganism, as several strains can cause disease when administered to axenic IL-10$^{-/-}$ mice. Another very important feature of this mouse model is an increase in intestinal permeability. Because several inflammatory mediators can cause the intestinal mucosa to leak, it has been difficult to establish whether the defect in intestinal permeability occurs independently of inflammation or whether it is a product of it. The results shown in this chapter demonstrate that increased intestinal permeability can occur well before the increase in proinflammatory cytokines, preceding colonic disease by several weeks in this mouse model. Interestingly, the defect in intestinal permeability in this animal model is initially in the small intestine, an organ that, at least in our IL-10$^{-/-}$ mice colony, does not exhibit histological inflammation.

These findings raised several important questions. First, why is there no inflammation in the small intestine of IL-10$^{-/-}$mice? Although this was not tested experimentally during my doctoral work, one could speculate that it is because of

two important characteristics of the small intestine: its physiological conditions and its reduced microbial flora (compared to the colon). The small intestine has the essential physiological functions of digesting and absorbing nutrients. These complex processes are in part achieved by a heavy secretion of fluid into the intestinal lumen. Unlike the small intestine, one of the colon's main functions is to reabsorb a majority of this fluid and this is achieved by a decrease in the transit time of the luminal contents in the colon. This much longer transit time in the colon could help explain how this section of the intestinal system is more prone to exhibit inflammation. A longer transit time could allow for a prolonged interaction of more concentrated luminal contents with the colonic mucosa, increasing the opportunities for inflammation to develop in a predisposed host. Another important difference between the colon and the small intestine that may explain why disease rarely occurs in the IL-$10^{-/-}$ mouse model is the amount and the type of microbial flora that each tissue harbours. The colon possesses ideal physiological conditions for microorganisms to adhere, survive and multiply in very high numbers. Although present, the microbial flora in the first two thirds of the small intestine is not as heavily populated or as biodiverse as the distal ileum or the colon (17), which may result in less infection foci and consequently less inflammation in the small intestine in these animals. In humans, CD manifest mainly in the lower portions of the ileum and/or the colon, possibly also because of the higher microbial load in these sections of the intestine.

Another question that remains is whether this permeability defect is a primary defect or if it is a consequence of the interactions of certain types of microorganisms with the intestinal mucosa. This could be easily addressed by measuring intestinal permeability in germ- free mice at different time points. Although I initially proposed to perform these experiments, our colony was contaminated several times during the course of my degree and I was not able to do so. Some of this work was done by Madsen *et al* and it showed that IL-10$^{-/-}$ mice raised under germ-free conditions do not have increased ileal or colonic permeability compared to wild-type mice (7). Thus, it is likely that the defect in small intestinal permeability in these mice develops as a consequence of bacterial interactions with the intestine, although the possibility exists that the epithelial barrier's susceptibility to bacteria is due to inherent defects in the intercellular junctions in this mouse model.

Finally, it still needs to be addressed whether there is a direct correlation between the defect in small bowel permeability and colonic disease, and if so, how is it that a defect in the small intestine leads to inflammation in the colon? These two questions were addressed experimentally and are discussed in the following chapters of this thesis.

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Chapter 3

Increased intestinal permeability as a requirement for colitis in the IL-10$^{-/-}$ mouse[3]

3.1 Introduction

Increased intestinal permeability has been proposed as a cause of systemic disease for decades. In several autoimmune diseases such as diabetes, CD and celiac disease, increased intestinal permeability has been recognized as an early feature of the disease. In CD, increased intestinal permeability has been described in non-inflamed portions of the gut, as well as in healthy first degree relatives of patients, suggesting that this defect can occur independently of inflammation (1-5). A defect in intestinal permeability prior to the onset of inflammation has also been described in several animal models for IBD, including the IL-10 gene deficient (IL-10$^{-/-}$) mouse, the SAMP mouse, and the mdra1a$^{-/-}$ mouse (6-8). However, whether this is an epiphenomenon, an early manifestation of disease or a critical step in disease pathogenesis is unknown and has been the subject of much debate.

The regulation of paracellular traffic across the intestinal epithelial barrier occurs at the junctions between epithelial cells. Closest to the luminal side is the tight junction, a complex structure composed of protein fibrils that connect a cell's actomyosin ring to the protein fibrils of the adjacent cell. The tight junction opens and closes in response to intra and extra cellular signals. These signals can be produced by the host (9-11) or by the bacteria in the lumen. (12;13) The

[3] *Part of this chapter has been published as a research paper: Arrieta et al, 2009. Gut;58(1):41-48.*

bacterium *Vibrio cholerae* expresses a battery of toxins as virulence factors. One of them, the ZOT, opens the paracellular space of the small intestinal epithelium by binding to an apical receptor and redistributing the F-acting cytoskeleton via activation of protein PKC. (14;15)

Synthetic peptides corresponding to amino acids in the receptor-binding motif of ZOT have been synthesized as a ZOT agonist (AT-1002) and antagonist (AT-1001). AT-1002 is a hexamer-peptide that reversibly decreases transepithelial resistance by rearranging the actin cytoskeleton via activation of src and mitogen activated protein (MAP) kinase pathways (16). AT-1001, also known as larazotide acetate, prevents the increase in paracellular permeability by competitively blocking the apical ZOT receptor and preventing the opening of tight junctions (17). This is believed to only occur in the small intestine as the ZOT/zonulin receptor has been found in the jejunum and distal ileum, but not in the colon (15).

The development of these ZOT-derived peptides has allowed us to study *in vivo* the role of small intestinal permeability in the pathogenesis of colitis in IL-$10^{-/-}$ mouse. This mouse develops a patchy, chronic colitis similar to human CD (18). From the point of view of this study, the IL-$10^{-/-}$ mouse model of disease has two important features. Firstly, these animals have been described as having increased small intestinal permeability from very early in life and well before the onset of disease (19). Secondly, disease development is dependent upon luminal factors: it does not occur in animals raised under germ free conditions (6;20). These observations suggested that the colitis observed in these animals might

develop as a consequence of abnormal small intestinal permeability with increased presentation of a luminal agent to the mucosal immune system. This is a hypothesis that we have previously proposed for several other autoimmune diseases (21).

Perhaps the best evidence suggesting that increased intestinal permeability has an etiological role in autoimmune disease comes from the BB rat model of diabetes. In these animals autoimmune Type 1 diabetes develops spontaneously in animals fed a normal diet, but can be prevented with a hydrolyzed diet. We have previously demonstrated that these animals have increased gastric and small intestinal permeability even when receiving a hydrolyzed diet (22). Furthermore, the expression of diabetes in animals consuming a chow diet can also be prevented by abolishing the increase in intestinal permeability with the ZOT receptor antagonist AT-1001 (23). These data strongly suggest that, at least in this animal model of autoimmune disease, dietary antigens can initiate disease through a mechanism that involves increased gastrointestinal permeability. It also provides support for the concept that there may be novel means of preventing some autoimmune diseases by targeting increases in gastrointestinal permeability.

Therefore the goals of this study were to determine whether the previously reported leakiness of the small intestine could be modulated by treating IL-$10^{-/-}$ animals with AT-1002 and AT-1001. By doing so, we were able to determine whether decreasing small intestinal permeability prevents or ameliorates colitis, and conversely, whether increasing it worsens disease.

3.2 Materials and Methods

3.2.1 Animals

Homozygous IL-10$^{-/-}$ mice generated on a 129 Sv/Ev genetic background, and 129 Sv/Ev controls were housed under specific pathogen-free conditions until weaning (3 weeks), when they were moved to conventional animal housing. The mice were housed in HEPA filter cages and fed a standard mouse chow diet. These mice were bred and raised in the animal facility at the University of Alberta. All animal procedures were conducted in accordance with protocols approved by institutional Animal Care and Use Committees.

3.2.2 Pharmacological therapy

3.2.2.1 AT-1001 treatment

Three and a half week old IL-10$^{-/-}$ mice and 129 Sv/Ev controls were randomized into four groups (n=10-13 for each group). Starting at 4 weeks of age, 2 groups of IL-10$^{-/-}$ mice were treated with AT-1001 in their drinking water, whereas 1 group of

IL-10$^{-/-}$ mice and 1 group of 129 Sv/Ev mice did not get the treatment. The drinking water of the two treatment groups was prepared daily by dissolving either 0.1 mg/ml (low dose) or 1.0 mg/ml (high dose) of AT-1001 in filtered distilled water. Placebo groups drank filtered distilled water prepared daily as

well. Mice were treated until 17 weeks of age, when animals were sacrificed by cervical dislocation.

3.2.2.2 AT-1002 treatment

For the acute experiments with wild type mice, 129 Sv/Ev mice aged 8-9 weeks were randomized into 6 groups (n = 3-6 per group). These mice were orally gavaged with 0.2 ml of a sugar probe and individually placed in metabolic cages twice a week for 24 hrs over a period of two weeks for urine collection, in order to acclimatize them to the *in vivo* permeability measurement technique. After each 24 hr period in metabolic cages mice were returned to their conventional housing. The permeability measurement done during the last acclimatization in metabolic cages was used as the basal permeability measurement. Twenty-four hours after the basal measurements, mice were gavaged with 0.1 ml of increasing concentrations of AT-1002 (0.0025, 0.025, 0.25, 1.25, 2.5 mg/g) and 0.2 ml of sugar probe, or 0.1 ml bovine serum albumin (BSA; 0.25 mg/g) and 0.2 ml sugar probe. Immediately after the gavage, mice were placed in metabolic cages for 24 hr for urine collection, during which time they also received AT-1002 or bovine serum albumin (BSA) in drinking water, at the same concentrations as the oral gavages. Animals were returned to their conventional cages for a 24 hr recovery period and placed in metabolic cages once again to measure their permeability post-drug treatment.

For the chronic experiment with IL-$10^{-/-}$ mice, animals aged 3.5 weeks were randomized into 2 groups (n = 9-11 per group). One group of IL-$10^{-/-}$ mice received AT-1002 in drinking water, whereas the other group of IL-$10^{-/-}$ mice received vehicle. The drinking water was prepared daily by dissolving 1 mg/ml of AT-1002 in filtered distilled water. The placebo group drank filtered distilled water as well. Treatment continued until 12 weeks of age, when animals were sacrificed by cervical dislocation. Permeability measurements were performed once every week.

AT-1001 and AT-1002 were graciously provided by ALBA Therapeutics (Baltimore, Maryland) as a powdered chemical.

3.2.3 In-vivo permeability measurement

Mice were housed in metabolic cages after a 4 hour fast of food and water and immediately after a gavage of 0.2 ml of a sugar probe containing 100 mg of sucrose, 12 mg of lactulose, 8 mg of mannitol and 6 mg of sucralose. After the collection of urine the animals were placed in their respective cages, and provided with food and water.

Urine from each animal was collected for 22-24 hours in collection tubes treated with 100 µL of a 10 % Thymol solution (1.0 g/ 10 mL isopropanol) and 100µL paraffin oil (to prevent urine evaporation). Samples were frozen at -70ºC until analysis. All sugars were quantified by ion exchange HPLC as previously described (24). Briefly, cellobiose was added as an internal standard, and the urine

was filtered through a 0.4-μm filter and diluted as necessary. Samples were deionized and then injected on a Dionex MA-1 ion exchange column. Sugars were eluted with NaOH at a flow rate of 0.4 ml/min. Peaks were detected using pulsed amperometric detection on a Dionex HPLC and quantitated as peak areas. Final data were reported as either fractional excretions (sucrose and sucralose) or as a ratio of fractional excretions (lactulose-mannitol). Fractional excretion is defined as the fraction of the gavaged dose recovered in the urine sample.

Sucralose was also assayed by HPLC. Separation was achieved using a Dionex Ionpac NS1 column and acetonitrile-water as the eluent at a flow rate of 1 ml/min. Detection was performed with an electrochemical detector in a fashion identical to the other sugars. For these assays, the internal standard used was phenyl-β-D-thiogalactoside (Sigma Chemical) added to the initial urine sample at a concentration of 0.1 mg/ml.

3.2.4 Ussing chamber assay

Mice were euthanized at 8 weeks of age by cervical dislocation and a segment of colon removed. The mucosa was mounted in Lucite chambers exposing mucosal and serosal surfaces to 10 ml of oxygenated Krebs buffer (in mM: 115 NaCl, 8 KCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 2 KH_2PO_4, 225 $NaHCO_3$; pH 7.35). The buffers were maintained at 37°C by a heated water jacket and circulated by CO_2/O_2. Fructose (10 mM) was added to the serosal and mucosa sides. For measurement of basal mannitol fluxes, 1 mM of mannitol with 10 μ:Ci [H^3] was added to the

mucosal side. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (Isc) with an automatic voltage clamp (DVC 1000 World Precision Instruments, Sarasota, FL). Tissue ion resistance (1/G) was calculated from PD and Isc according to Ohm's Law (25).

3.2.5 Mucosal Cytokine and IgA secretion

At 17 weeks of age (AT-1001 treatment) or 12 weeks of age (AT-1002 treatment), small intestinal and colonic organ cultures were prepared from placebo and AT-1002 treated IL-10 $^{-/-}$ mice. Because of the patchy nature of colitis in IL-10$^{-/-}$ mice whole colons were removed, flushed with PBS, and ¼ of the tissue was cut longitudinally and resuspended in tissue culture plates (Falcon 3046; Becton Dickinson Labware, Lincoln Park, NJ) in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml), Cultures were incubated at 37°C in 5% CO_2. After 24 h, supernatants were harvested and stored at −70°C for analysis of cytokine levels. Pellets were left to dry for 4 days and weighed. IgA, TNF-α and IFN-γ levels in cell supernatants were measured using ELISA kits (Medicorp, Montreal, Quebec). Samples for IgA determination were diluted 1:100.

3.2.6 Myeloperoxidase (MPO Assay)

At 17 weeks of age (AT-1001 treatment) or 12 weeks of age (AT-1002 treatment), a section of the colon of all animals was removed upon dissection, weighed and immediately flash-frozen. Samples were kept at −70°C until determination of MPO concentrations. Levels of MPO in colonic tissue were measured using an ELISA kit (Cell Sciences Inc, Canton, Massachusetts).

3.2.7 Histologic Injury Grading

Upon sacrifice, ~ 7-9 cm of proximal, mid, distal ileum and the whole colon were harvested and fixed in 10% phosphate-buffered formalin. The same regions were dissected from each mouse. These samples were embedded in paraffin *in toto*, sectioned at 4 μm, and stained with H&E for light microscopic examination. The slides were reviewed in a blinded fashion by a pathologist (J.S.D.) and were assigned a histological score for intestinal inflammation using a scheme modified from Saverymuttu *et al* (26).

3.2.8 Statistical Analysis

Normally distributed data are expressed as means ± SEM. Comparisons between groups were made using ANOVA with a Tukey test for post hoc comparisons. All calculations were performed using Graphpad Prism 3.0 (San Diego, CA), and significance was assumed at $P < 0.05$.

3.3 Results

3.3.1 Effect of AT-1001 in small intestinal permeability and colitis in IL-10 $^{-/-}$ mice

When given daily in the drinking water the ZOT antagonist AT-1001 was effective in decreasing small intestinal permeability in the IL-10$^{-/-}$ mice (Fig. 3.1). After 4 weeks of treatment, high dose AT-1001 reduced small intestinal permeability back to levels observed in control animals. This was evident from 8 weeks of age (Fig. 3.1A). The animals treated with low dose AT-1001 showed a significant reduction in overall permeability (as expressed as the area under the permeability curve) as compared to the placebo group (Fig 3.1B), but this dose did not quite return small intestinal permeability to normal levels (Fig 3.1A).

The effect of AT-1001 in reducing small intestinal permeability was also evident *in vitro* at 8 weeks of age. As demonstrated in figure 3.2, small intestinal permeability to mannitol and electrical resistance were determined in Ussing chambers. Both doses of AT-1001 effectively reduced permeability to mannitol in the small intestine (Fig. 3.2A). Furthermore, the loss of electrical resistance, observed in the IL-10$^{-/-}$ animals, was prevented in the high dose treated group, but not in the low dose treated group (Fig. 3.2B).

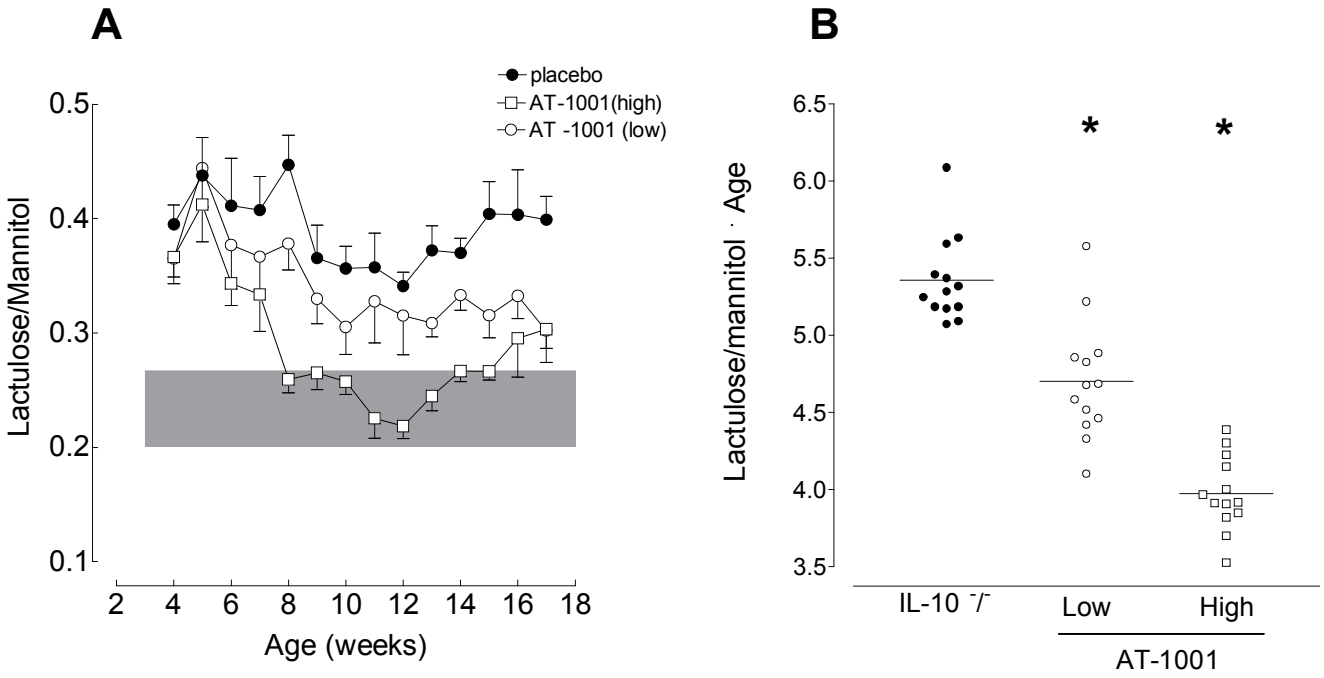


Figure 3.1 . Small intestinal permeability is reduced with AT-1001. A) Small intestinal permeability was measured in IL-10$^{-/-}$ mice treated with high dose AT-1001 (open squares), low dose (open circles) or placebo (closed circles) from 4 to 17 weeks of age. The shaded area represents the mean ± 3 STD of the lactulose/mannitol ratios observed in wild type mice. Mice treated with the high dose of AT-1001 eventually reached the wild type range. B) Statistical analysis of the areas under the curve of small intestinal permeabilities for each group. The permeabilities of the groups treated with AT-1001 differed significantly from the placebo group; $p < 0.05$ (low dose), $p < 0.01$ (high dose), n=10-13.

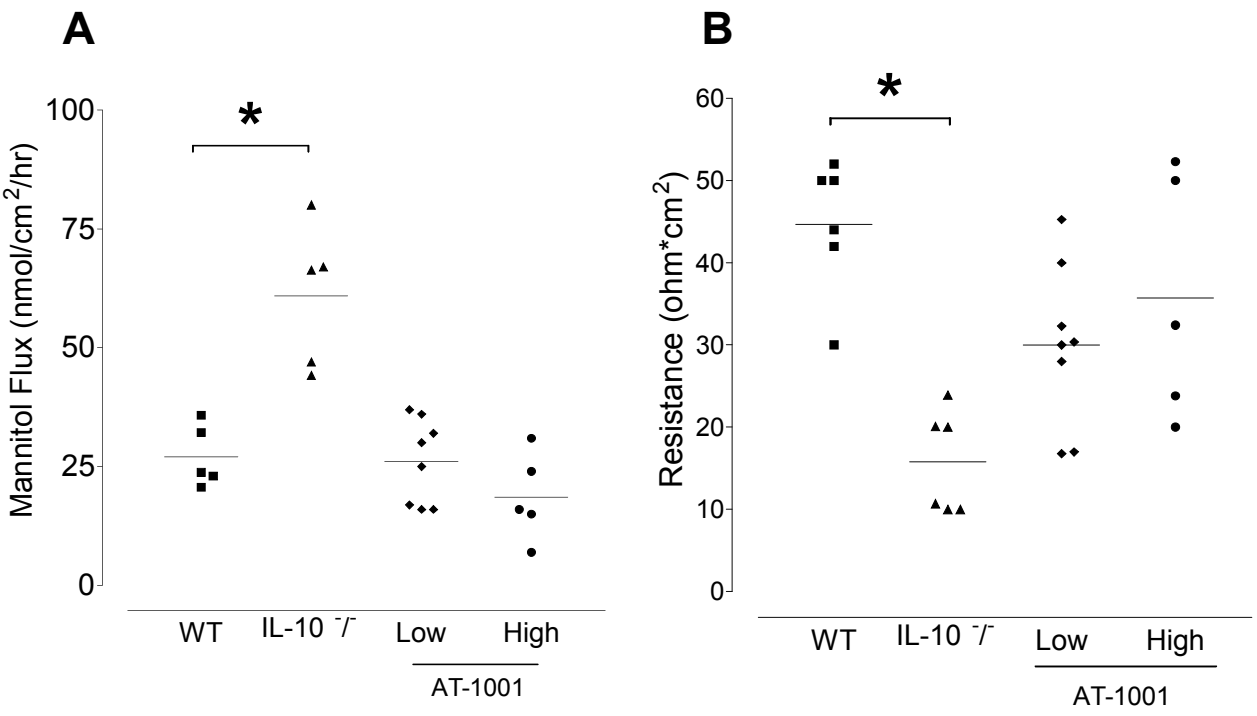


Figure 3.2 AT-1001 reduced small intestinal permeability by 8 weeks. A) Mannitol flux was measured in Ussing chambers at 8 weeks of age and was increased in the IL-10$^{-/-}$ mice ($p < 0.05$). This increased was not observed in animals treated with AT-1001 (n=4-8). B) The electrical resistance was reduced in the IL-10$^{-/-}$ mice only ($p < 0.01$). AT-1001 treatment prevented this reduction (n=5-8).

To test whether or not decreasing small intestinal permeability would affect the development of colonic disease, we evaluated several parameters of colonic disease. Over the entire course of the experiment we non-invasively determined epithelial colonic damage using the clearance of orally administered sucralose. Additional markers of colitis included *in vitro* colonic permeability at 8 weeks of age, and at 17 weeks of age, colonic cytokine secretion, MPO content and histology.

At 8 weeks of age colonic permeability to mannitol was increased in the IL-$10^{-/-}$ animals with a corresponding decrease in electrical resistance. In the treated animals these changes were largely prevented and both treated groups showed similar results to wild-type mice (Fig. 3.3A and B). This suggests that the early evidence of colonic disease, observed in IL-$10^{-/-}$ mice, is prevented or delayed by treatment with AT-1001.

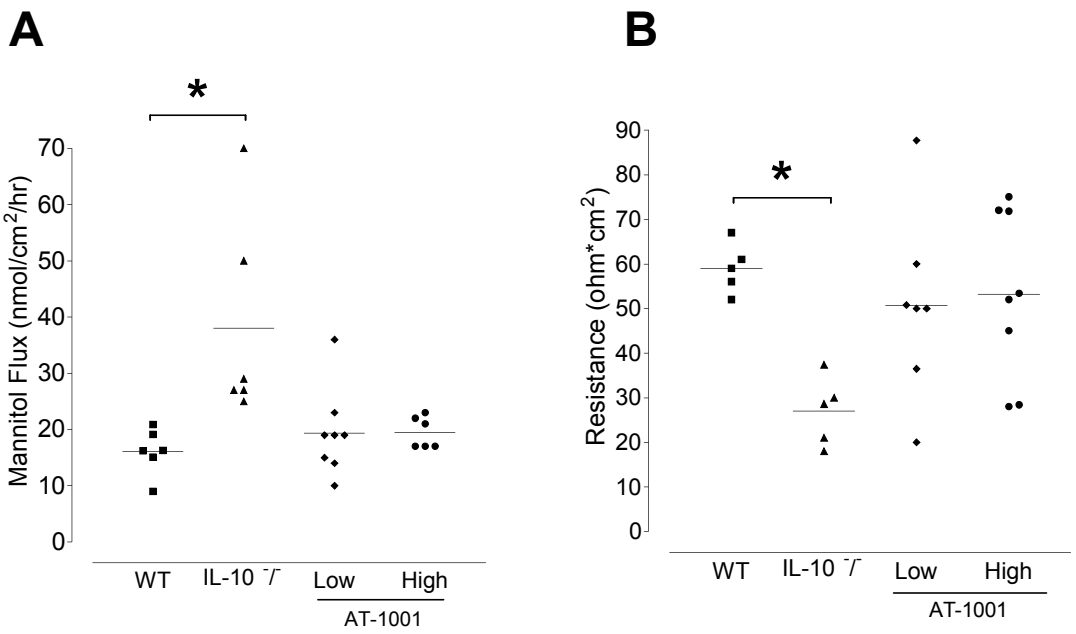


Figure 3.3 Attenuation of colonic permeability at 8 weeks. A) Colonic mannitol flux was increased in the IL-10$^{-/-}$ mice ($p < 0.05$). This increase was prevented by AT-1001 (n=5-8). B) Electrical resistance was reduced in the IL-10$^{-/-}$ mice and again prevented by AT-1001 ($p < 0.05$, n=5-8).

Colonic damage was measured weekly using sucralose excretion (Fig 3.4). IL-10$^{-/-}$ mice and controls excreted similar amounts of sucralose in their urine until week 10, following which they started showing elevated amounts of sucralose (Fig 3.4A). IL-10$^{-/-}$ animals had a significantly increased cumulative amount of damage as compared to the wild type animals (Fig 3.4B). Both treated groups had significantly less damage than the IL-10$^{-/-}$ animals but still significantly more than the wild type group. Treatment with AT-1001 appears to have significantly ameliorated, but not completely prevented colonic disease as determined by this parameter.

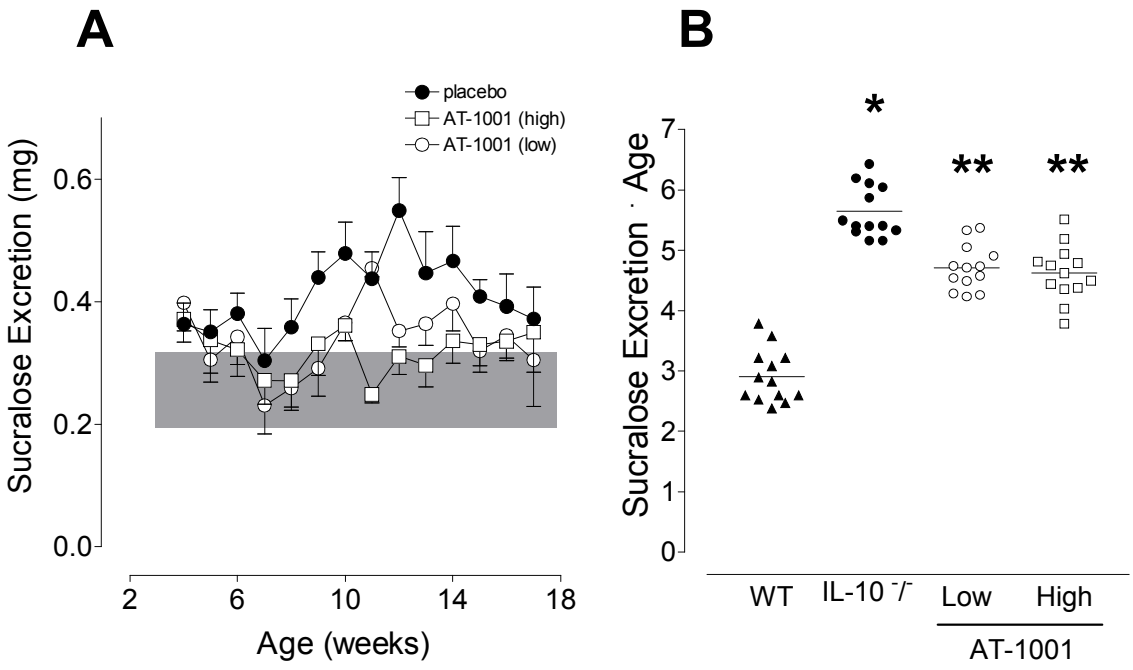


Figure 3.4 AT-1001 attenuates colonic damage. A) Sucralose excretion was measured weekly in IL-10$^{-/-}$ mice treated with a high dose of AT-1001 (open squares), low dose (open circles) or placebo (closed circles) from 4 to 17 weeks of age. The data is shown relative to that from wild type animals, which is represented in the shaded area. B) Statistical analysis of the areas under the curve of sucralose excretion for each group. (* $p < 0.01$; ** $p < 0.05$, n=10-13), suggesting that AT-1001 reduces but does not prevent colonic damage.

At 17 weeks of age, IFN-γ and TNF-α secretion in the colon was clearly reduced in the groups treated with AT-1001. Only the IL-10$^{-/-}$ group showed significantly increased rates of cytokine secretion compared to the wild-type animals (Fig 3.5A and B). Neutrophil infiltration in the colon, as measured by tissue MPO content (Fig 3.5C), was increased in the IL-10$^{-/-}$ and low dose AT-1001 mice as compared to controls but was not significantly increased in the high dose treated group. Taken together, these data suggest that treatment with AT-1001, active in the small intestine was effective at reducing the colonic inflammation observed in these animals.

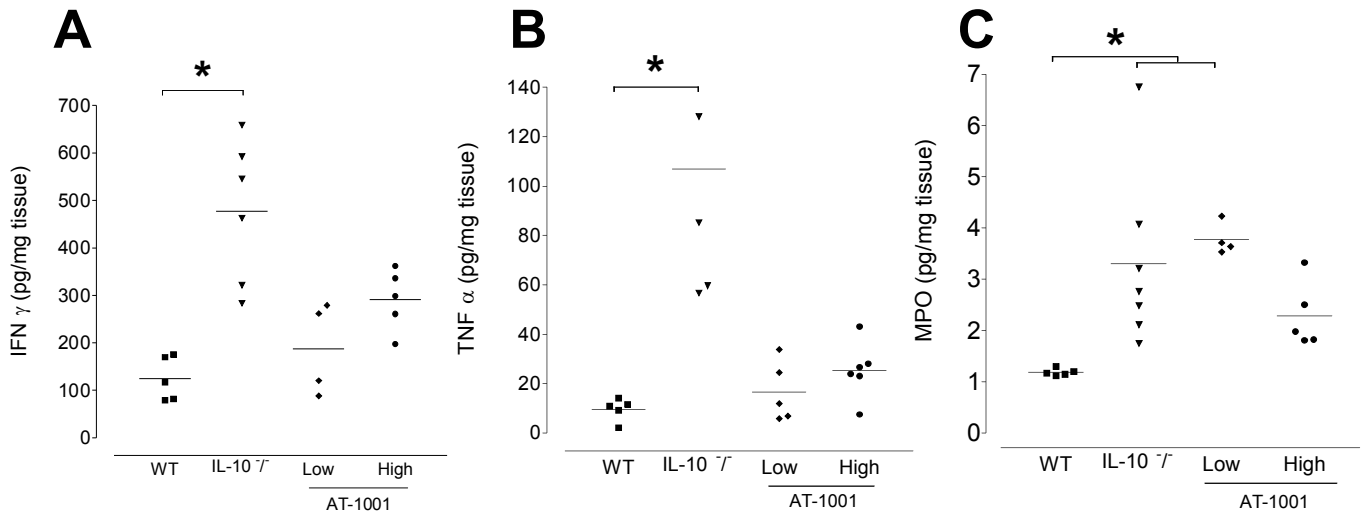


Figure 3.5 Colonic secretion of IFN-γ, TNF-α and MPO content at 17 weeks. A and B) The IL-10 $^{-/-}$ mice had a significant increase in colonic secretion of these cytokines ($p < 0.05$). This increase was prevented by AT-1001 treatment (n=4-6). C) The IL-10 $^{-/-}$ mice and the mice treated with the low dose had a significant increase in MPO content ($p < 0.05$). High dose treatment of AT-1001 prevented this increase (n=4-6).

A similar attenuation effect was observed histologically. These results are illustrated in figure 3.6. Significant inflammation was observed in the IL-$10^{-/-}$ mice that was similar to that observed in those treated with the low dose of AT-1001. However, those animals treated with the high dose of the compound had a significant reduction in this histological score, although it was still significantly greater than the scores observed in the control animals.

Representative examples of the colonic histology are presented in fig 3.7. The upper panels are taken at 20X magnification whilst the lower are at 40X. Panels A/B illustrate the histological features observed in the background strain of animals with only a sprinkling of inflammatory cells in the lamina propria. This is in contrast to the IL-$10^{-/-}$ mice shown in Panels C/D where there is a marked increase in the inflammatory cell compartment. Panels E/F and G/H are representative sections taken from animals treated with the low and high doses of AT-1001 respectively. It can be appreciated that the inflammatory changes observed in these animals are less than that seen in the IL-$10^{-/-}$ mice.

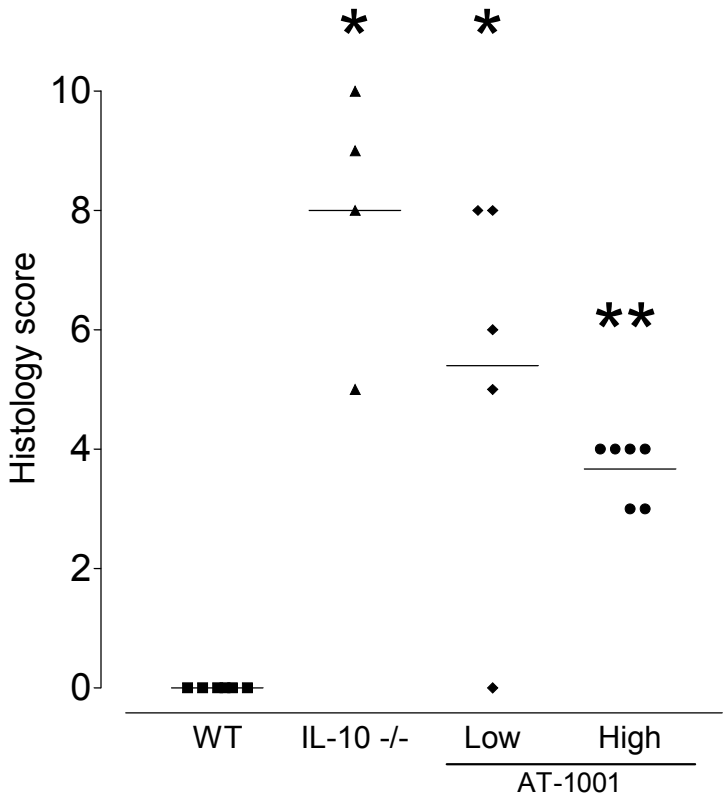


Figure 3.6 Histologic scoring. The IL-$10^{-/-}$ mice and the animals treated with a low dose AT-1001 had significantly increased histological scores as compared to the wild type mice (* $p < 0.05$). However, the high dose AT-1001 treated group had a histological score significantly lower than the untreated IL-$10^{-/-}$ animals (** $p < 0.05$), but this was still elevated significantly from the wild type controls (n=4-6).

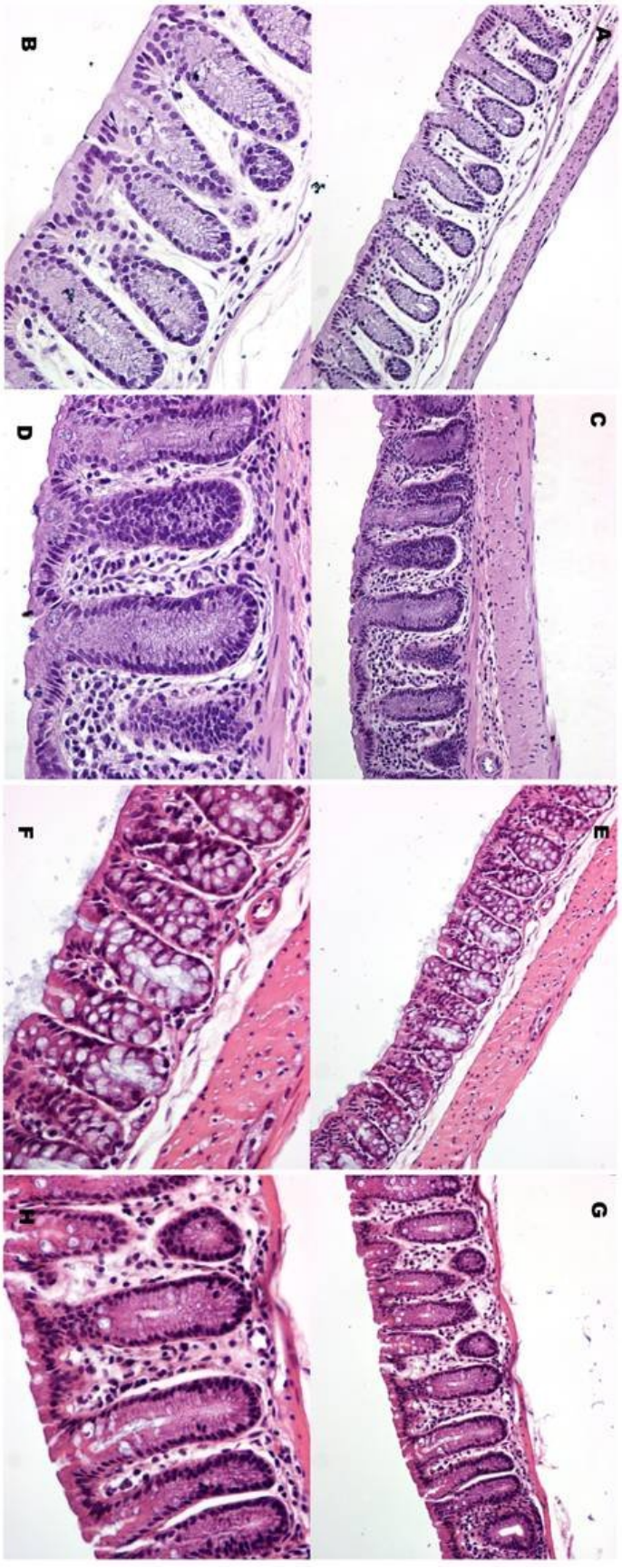

A
B
C
D
E
F
G
H

Figure 3.7 Representative histology. All panels show colonic histology at 17 weeks of age. The upper panels are pictures taken at 20X magnification whilst the lower are the same regions at 40X. Panels A/B represent tissue from the wild type animals while panels C/D are from the IL-$10^{-/-}$ mice. A clear inflammatory infiltrate is observed in the lamina propria of these animals. Panels E/F are from the low dose group while G/H are from the high dose treated animals. It can be appreciated that there is a significant reduction in the inflammatory component of the lesion observed in these animals.

It has been suggested that the ZOT receptor is absent in the colon, however, this work was done in rabbits, not mice (15). Therefore, to rule out the possibility that this pathway is present in the colon and that the effects observed throughout our experiments were a consequence of the drug in the colon, we performed a functional assay to test for the presence of a permeability effect in the colon using a ZOT peptide agonist (AT-1002). As observed in figure 3.8 after challenge with this agent, only small intestinal permeability was increased, while sucralose excretion stayed the same. This was true for both the IL-$10^{-/-}$ and wild type mice. Using increasing concentrations of AT-1002 we were able to demonstrate that the increase in lactulose/mannitol ratio was dose dependent and at 0 dose, up to the solubility limit of AT-1002, did we observe any effect in the colon (data not shown). When both wild-type and IL-$10^{-/-}$ animals were treated with a combination of AT-1001 and AT-1002, the increase in small intestinal permeability was abolished, demonstrating that the effect of AT-1002 to increase small intestinal permeability could be prevented or blocked by the same agent we used in the previous experiments.

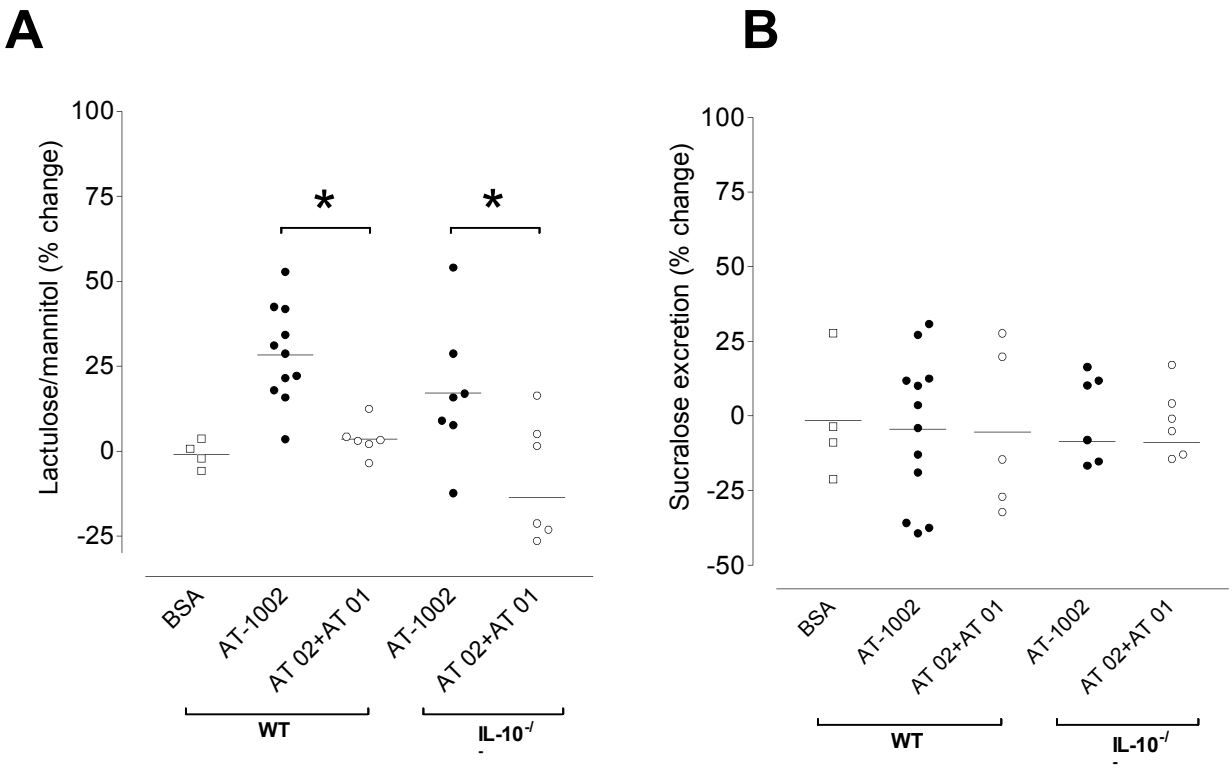


Figure 3.8 ZOT-mediated changes in permeability. A) Mice were treated with AT-1002 (1mg/ml) or a mixture of AT-1002 and AT-1001 (1mg/ml each) for 24 hrs. AT-1002 increased small intestinal permeability (lactulose/mannitol ratio). The concomitant administration (AT 01 + AT 02) abrogated this increase. The same effect was demonstrated in IL-$10^{-/-}$ animals. BSA was used as a protein control ($p < 0.05$; n=4-12). B) Colonic permeability (sucralose excretion) was determined in the same animals. In contrast to small intestinal permeability, stimulation of the ZOT pathway had no effect in the colon of either wild type or IL-$10^{-/-}$ mice (n=4-12).

3.3.2 Effect of AT-1002 on intestinal permeability and inflammation in wild type mice

In order to test whether the tight junction modulator AT-1002, had an *in vivo* effect similar to what had been demonstrated *in vitro,* (27) increasing concentrations of the drug where given by oral gavage and in drinking water to wild type mice. The mice had their basal intestinal permeability measured, followed by a 24-hour administration of the drug and a second permeability measurement. Finally, a third permeability measurement was performed 24 hours after the drug AT-1002 had been removed from the animals' drinking water.

A rapid change in small intestinal permeability, measured as the percentage change in the ratio of the fractional urinary excretions of mannitol and lactulose (lacman ratio), was detected in the animal groups that received the three highest doses of AT-1002 (fig 1A). The animals that received the two highest doses (1.25 and 2.5 mg/g) showed signs of stress (reduced motility, lack of grooming) and diarrhea, so all subsequent experiments in IL-10 $^{-/-}$mice were performed using an effective but lower dose (0.25 mg/g), which induced a ~30% change in lacman ratio during the 24-hour drug challenge. The effect of AT-1002 was also found to be short lived, as the animals returned to their basal small intestinal permeability within 24 hours after the drug had been removed from the drinking water (fig 3.9)

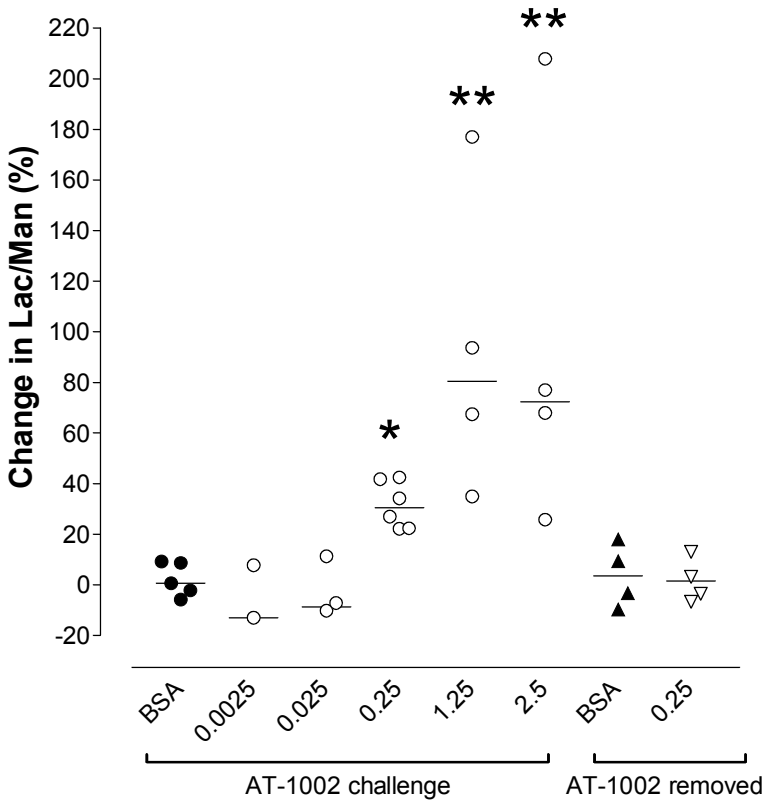
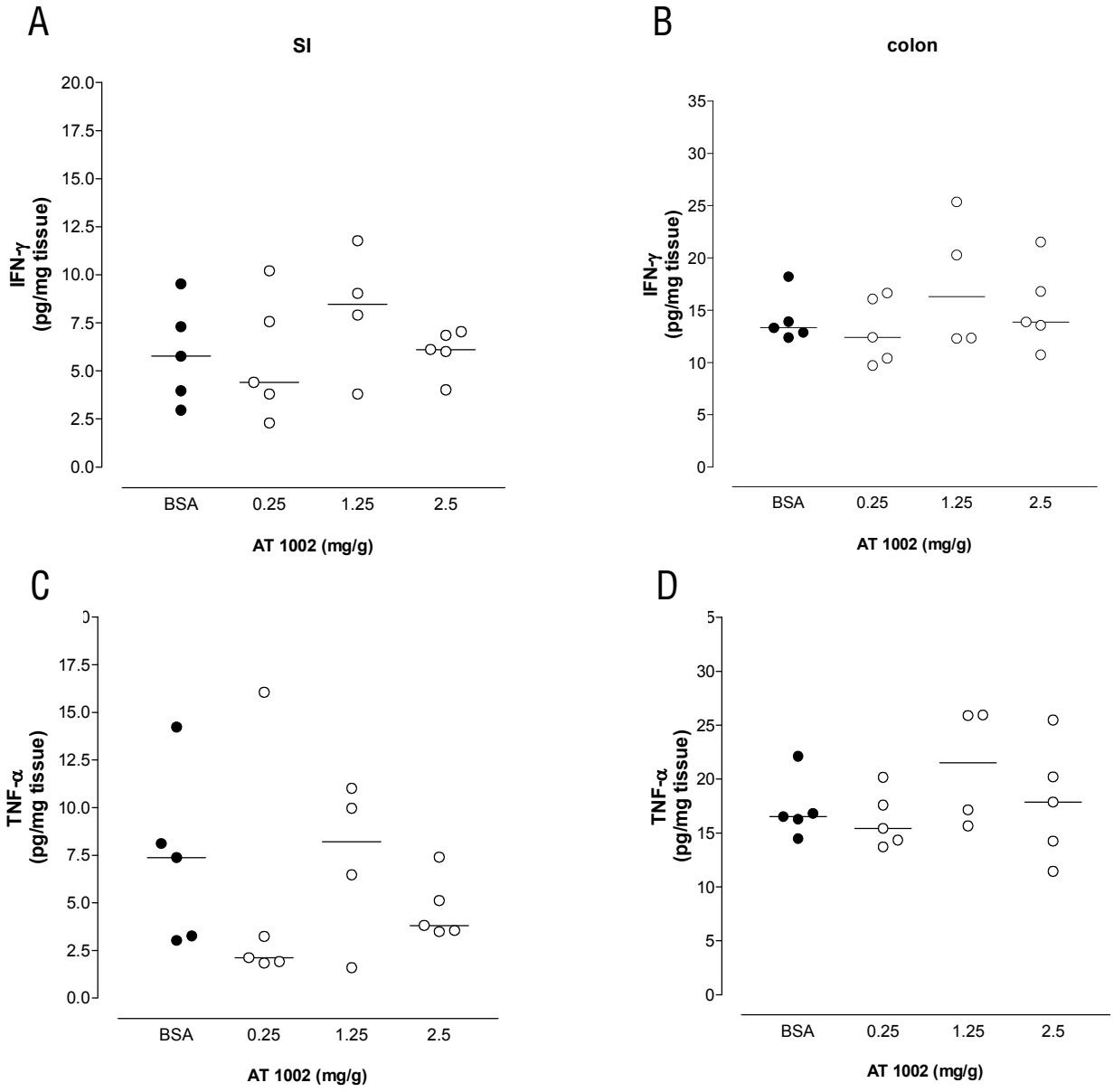


Figure 3.9 Effect of AT-1002 in the small intestinal permeability of wild type mice. Percentage change in lacman ratio between the basal permeability measurement and the day of AT-1002 treatment, or 24 hrs after the removal of AT-1002. AT-1002 had a rapid and dose dependent effect in small intestinal permeability. The effect was short lived as small intestinal permeability returned to basal levels 24 h after AT-1002 was removed. * $p<0.05$; ** $p<0.01$, n = 3-6.

In order to determine whether increasing small intestinal permeability would induce inflammation in wild-type mice, mice given the three highest doses of AT-1002 were sacrificed 96 hours after the drug was administered. Small intestinal and colonic explants were cultured for 24 hours and IFN-γ and TNF-α secretion analyzed. AT-1002 did not induce any increase in these pro-inflammatory cytokines in either the small intestine or the colon, (fig 3.10 A-D), suggesting that the rapid increase in small intestinal permeability caused by AT-1002 does not acutely induce an inflammatory response.


A
SI
IFN-γ (pg/mg tissue)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
BSA
0.25
1.25
2.5
AT 1002 (mg/g)
B
colon
IFN-γ (pg/mg tissue)
35
30
25
20
15
10
5
0
BSA
0.25
1.25
2.5
AT 1002 (mg/g)
C
TNF-α (pg/mg tissue)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
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BSA
0.25
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AT 1002 (mg/g)
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TNF-α (pg/mg tissue)
30
25
20
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10
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0
BSA
0.25
1.25
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AT 1002 (mg/g)

Figure 3.10 AT-1002 did not induce an acute inflammatory response in the gut. Secretion of IFN-γ (A and B) and TNF-α (C and D) from small intestinal (A and C) and colonic (B and D) explants of mice treated with increasing doses of AT-1002. No change in proinflammatory markers was observed 96 hours after the administration of AT-1002, n = 4-5.

3.3.3 Effect of AT-1002 in small intestinal permeability and colitis in IL-10 $^{-/-}$ mice

Although we had shown that AT-1002 was effective at increasing small intestinal permeability in wild type mice, it was unclear if it would have a similar effect in the IL-10$^{-/-}$ mice. As we have shown previously, this mouse model has a significant increase in small intestinal permeability as early as 4 weeks of age and it was unknown if the tight junction modulator AT-1002, could increase the already elevated small intestinal permeability in this mouse. To test this, IL-10$^{-/-}$ mice were given AT-1002 *ad libitum* in drinking water from 4 to 12 weeks of age. Weekly intestinal permeability measurements showed that AT-1002 was effective at increasing small intestinal permeability in IL-10$^{-/-}$ mice in most weeks, compared to the placebo group (fig 3.11A). Overall, AT-1002 caused a significant increase in small intestinal permeability, as expressed by the area under the permeability curve (fig 3.11B).

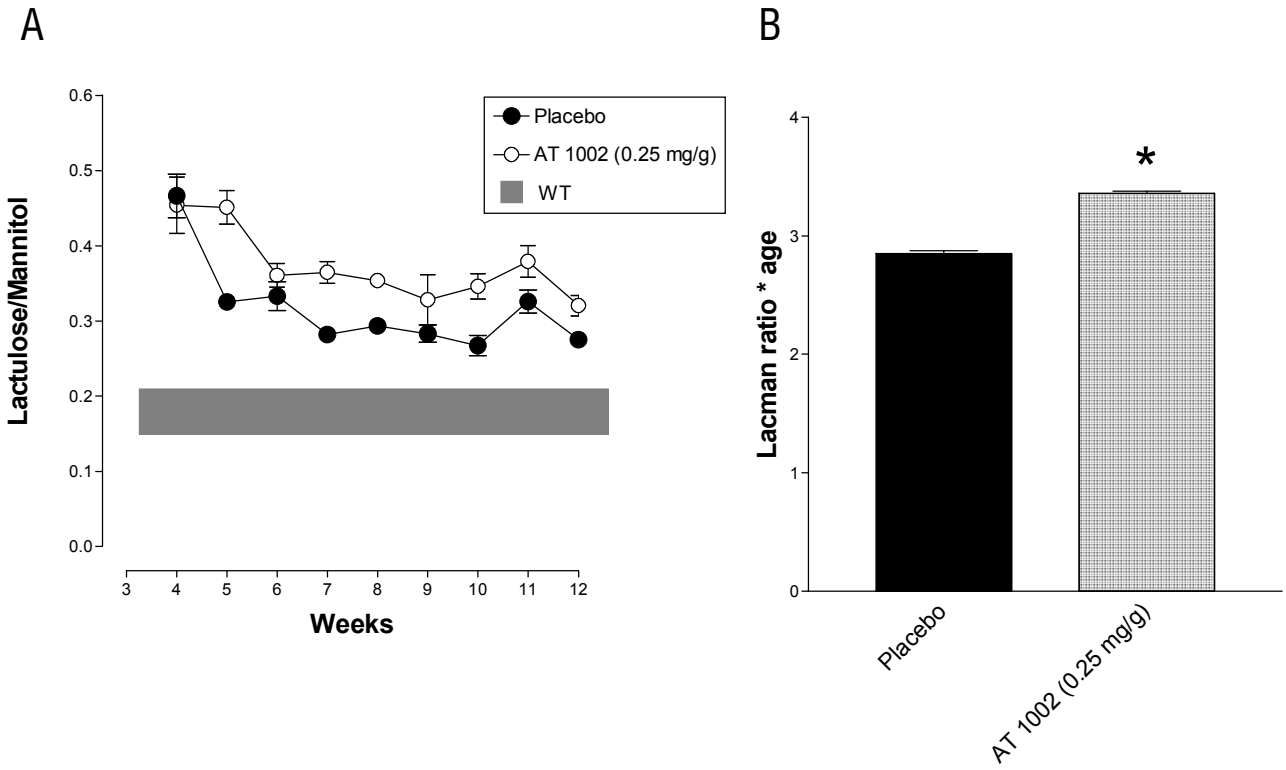

Figure 3.11 AT-1002 increased small intestinal permeability in IL-10$^{-/-}$ mice. A) Small intestinal permeability was measured weekly in IL-10$^{-/-}$ mice treated with AT-1002 in drinking water (open circles) and placebo (closed circles), between 4-12 weeks of age. The shaded area represents the mean (±3 SD) of the lactulose/mannitol ratios observed in wild type mice. B) Statistical analysis of areas under the curve of mice treated with AT-1002 treated or placebo. The small intestinal permeabilities from the AT-1002 treated group were significantly higher than the permeabilities of the control group; $p<0.01$, n = 9-11.

At 12 weeks of age, TNF-α secretion in the colon was significantly increased in the group treated with AT-1002 (fig 3.12A), although the difference in IFN-γ secretion did not reach statistical significance (fig 3.12B). Colonic MPO content, a marker of neutrophil infiltration, was also significantly increased in the group treated with AT-1002 (fig 3.12C). These data suggest that long term treatment with AT-1002 and the consequent increase in small intestinal permeability enhanced an inflammatory response in the treated animals.

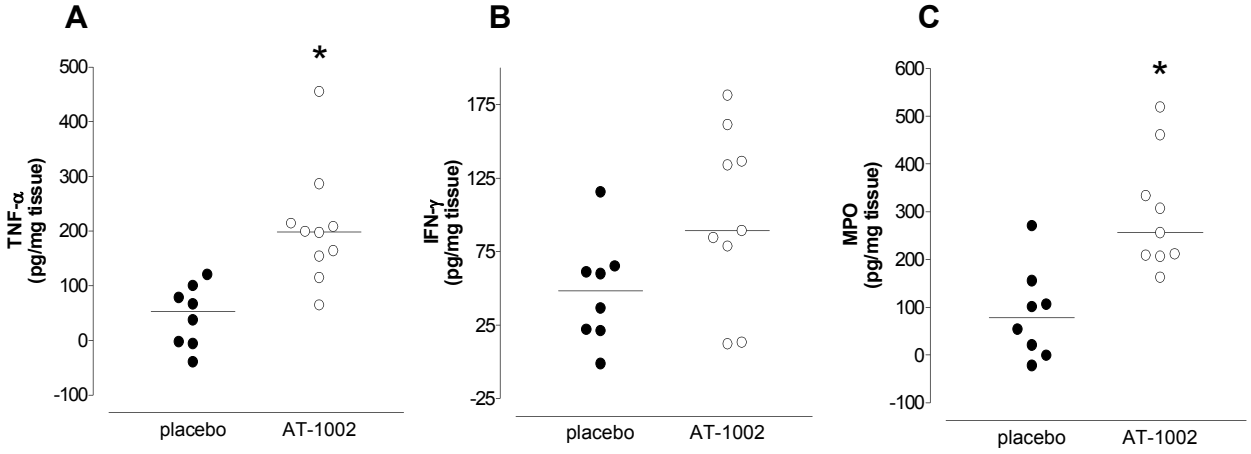

Figure 3.12 AT-1002 increased inflammation in IL-10$^{-/-}$ mice. Colonic secretion of TNF-α (A), IFN-γ (B) and (C) MPO from cultured tissue explants. Mice treated with AT-1002 showed a significant increase in TNF-α and MPO. The apparent increase in IFN-γ did not reach statistical significance; $p<0.01$, n = 9-11.

3.3.4 Effect of AT-1002 on IgA secretion

One of the main secretory products in the small intestine is secretory IgA (sIgA), a key immunoglobulin in the first line of defense of the epithelial barrier. Because AT-1002 showed such a marked effect in intestinal permeability in both wild type and IL-$10^{-/-}$ mice, we were interested in testing whether this increase in permeability was associated with an increase in total IgA secretion. To test this, small intestines were taken from the animals treated with AT-1002 and placebo during 12 weeks. Tissue explants were cultured for 24 hours and total IgA was measured in the supernatant. IL-$10^{-/-}$ mice treated with AT-1002 showed a significantly increased secretion of total IgA in the small intestine (fig 3.13).

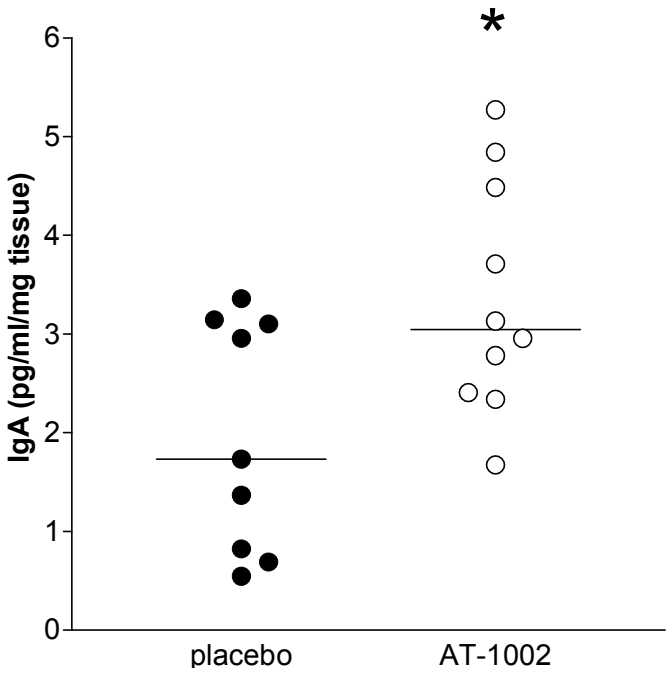

Figure 3.13 AT-1002 effect in IgA secretion. IgA was measured by ELISA in small intestinal explants of IL-10$^{-/-}$ mice treated with AT-1002 or placebo. AT-1002 significantly increased IgA secretion at 12 weeks; $p<0.05$, n = 9-11.

3.4 Discussion

The etiology of Crohn's disease is poorly understood. However, it has been proposed that three main features must be present for disease to occur: 1) a genetically susceptible mucosal immune system, 2) an antigen, or pro-inflammatory compound, that reaches the gut and can trigger the susceptible immune system and 3) an alteration in gut barrier function that allows this antigen to have contact with the mucosal immune system (21). In this regard the IL-10 deficient mouse is an interesting model of human Crohn's colitis. It does not develop colitis in the absence of intestinal bacterial flora, suggesting that one or more components within the enormously complex flora trigger the susceptible mucosal immune system to develop inflammation.

The experiments presented here provide evidence that a break in small intestinal barrier function is a crucial event in the outcome of colitis in the IL-10$^{-/-}$ mouse. This and other animal models of Crohn's disease, including the SAMP and the mdra$^{-/-}$ mouse have shown increased small intestinal permeability well before disease expression (6-8). This study extends these observations in an important manner by demonstrating that an increase in small intestinal permeability worsens the colonic inflammatory response. Other authors have also reported a similar association between an increase in intestinal permeability and the development of colitis in myosin light chain kinase (MLCK) transgenic mice. These mice express a constitutively active MLCK which disrupts the tight junction through cytoskeletal modification. While these mice do not spontaneously develop intestinal inflammation, they show subclinical inflammation in the intestinal

mucosa and an increase in susceptibility to the adoptive transfer of colitis-inducing T cells (28).

In our experiments with AT-1002 in wild type animals, we observed a fast and reversible effect of AT-1002, and further, that treatment with AT-1002 was effective in increasing the already elevated permeability in the IL-$10^{-/-}$ mice. This suggests that although the epithelium in these animals is leaky, the pathway by which AT-1002 opens the paracellular space is still active and can be stimulated to allow for even more passage of luminal material paracellularly. One of the possibilities we considered with the use of AT-1002 was that it could induce hypersecretion of fluid into the lumen and produce diarrhea; therefore we opted to first perform a dose response experiment with wild-type mice. It was evident that the two highest doses of the drug (1.25 and 2.5 mg/g) did cause diarrhea, therefore we chose to use the next effective dose, which did not induce such a drastic change in permeability but also did not cause diarrhea.

Another possibility that needed to be tested was that the drug might induce an inflammatory response on its own. Assessment of the small intestine and colon 4 days after administration of AT-1002 for 24 hours found no increase in any measured inflammatory marker. In contrast, administering AT-1002 chronically for 12 weeks in the colitis prone animals induced an increase in secretion of TNF-α and tissue MPO.

Perhaps the most relevant finding in our study is that a reversal of this barrier defect can attenuate the disease, implying that the increased permeability is not

simply an epiphenomenon but rather is an important etiological event. It is important to recognize that in our experiment with AT-1001 even though colitis was ameliorated, it was not abolished. Even in the treated animals there was still histological evidence of mild inflammation as well as MPO accumulation. There are several potential explanations for this. First, animals received treatment only from the age of 4 weeks and small intestinal permeability was not reduced to "normal" until 8 weeks of age. Since the animals are weaned from their mothers at 3-4 weeks of age it is difficult to initiate this therapy any earlier. However, we know that the permeability defect is present by two weeks of age (6) and this suggests that we did not completely remove this potential inflammatory stimulus. Second, it is entirely possible that the colonic disease in this animal model is not only generated by events that occur proximally in the small intestine, but also by direct interaction of the colonic bacterial flora with the IL-10 deficient immune system. Thus, reducing antigen presentation in the small intestine may not completely abrogate disease as bacterial components could still induce secretion of proinflammatory cytokines in the colon and perpetuate inflammation (11;29). Normal gastrointestinal permeability is tightly regulated through numerous mediators and signaling pathways. Recent work suggests that one pathway that regulates permeability involves ZOT. The identification of this pathway has also provided an opportunity for pharmacological manipulation of increased permeability associated with overactivity of this pathway. Because the ZOT receptor is not found in colonic epithelium (15), drugs that work via this mechanism should not have effects in the colon. To confirm that this was true in

our mice, we tested whether a ZOT-mediated event could be elicited in the colon under conditions identical to those used in our experiments. We used AT-1002, a peptide very similar to AT-1001 but an agonist of the ZOT receptor, and found that at a high concentration it could only increase small intestinal permeability. Moreover, when both the agonist and antagonist were given together the change in small intestinal permeability was prevented, suggesting that they act through the same pathway. These data suggest that the ZOT receptor is not present in the colon of mice (as described in the rabbit) but we cannot exclude the possibility that the peptides were simply degraded during their transit of the small intestine. However, regardless of which explanation is correct, these data suggest that the modulation of colitis severity observed in this study following treatment with AT-1001 or AT-1002 was due to its effect in the small intestine.

In conclusion we believe that these experimental data support the hypothesis that in a genetically predisposed host, increased small intestinal permeability can contribute to the induction of inflammatory disease. In the BB rat the disease is expressed in the pancreatic islet cells (22), and reducing the abnormal permeability that precedes this disease with AT-1001 can ameliorate the resulting diabetes (23). The current study has extended these observations by demonstrating that in the IL-$10^{-/-}$ mouse abnormal small intestinal permeability not only precedes the development of colitis but it is also etiologically important. Loss of intestinal barrier function is not merely an early manifestation of colitis; it is a key etiological factor of the disease and part of what determines when and how severe the disease will manifest.

The main question that remains is how an increase in paracellular permeability in the small intestine worsens colonic disease? One possibility is that by increasing small intestinal leakiness, material from the lamina propria can leak into the lumen and change the intestinal microbial flora by modifying the environmental conditions that establish the bacterial ecology in the gut. Another possible mechanism is that an increase in small intestinal permeability induces a change in antigen uptake and immune tolerance to oral antigens. Both of these possible mechanisms were tested and are discussed in the next two chapters.

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Chapter 4

Effect of increasing small intestinal permeability on the microbial colonic flora of IL-10$^{-/-}$ mice

4.1 Introduction

As it has been described in previous chapters, the complex microbial intestinal flora is believed to be the trigger for IBD and, under increased intestinal permeability conditions, bacteria gain access to the immune components of the intestinal mucosa. In chapter 3 it was shown that modulating intestinal permeability in the small intestinal has a direct effect in the inflammatory process in the colon. Thus, we hypothesized that increasing small intestinal permeability may have a direct role in determining the microbial flora of the colon, thereby modulating disease.

Our knowledge of the intestinal microbiota has grown enormously in the past decades. New advances in detection of bacterial, fungal and viral species have determined that the mammalian gut is one of the most densely populated ecosystems on Earth (1). Up to 10^{13} bacteria can be found in feces, which is 10-fold greater than the number of cells in the body and comprises 100-fold more genes than our 2.85 billion base pair genome. The total number of genes encoded in the gut microbiota is defined as the microbiome and it is known to have a profound effect in our metabolic activities (2).

At birth the gastrointestinal (GI) tract is sterile, but shortly after it acquires microbial flora from the environment, in an oral-to-anal direction. It is believed that the microbial flora is established very early in life and that it remains mainly unchanged thereafter under normal conditions. This idea is supported by gut flora studies performed in monozygotic twins that live in different places. This study showed striking similarities in the twins' flora even though they lived apart for the majority of their lives. Moreover, each twin did not share many similarities in terms of enteric flora with their respective spouses, indicating that genetics dominates over diet and environment in determining the development of a particular enteric flora. (3) A later study compared the enteric flora between pairs of monozygotic and dizygotic twins, as well as pairs of siblings. In this study it was shown that the microbiota comparisons between dizygotic twins and between siblings was as similar as that one between monozygotic twins, suggesting that not only the genetic background is important in determining the type of enteric flora, but also the environmental factors they share early in life, such as their mother's breast milk. (4)

The enteric flora can be divided in two populations: autochthonous (resident flora) and allochthonous (transient flora). The adherent flora exists in 'biofilms', which consist of layers of bacteria that adhere to the intestinal mucus layer, whereas the transient flora is not part of this biofilm and is a main component of feces. (5)

The entire GI tract is colonized by bacteria, but their numbers and types change depending on the GI tract section surveyed, which clearly indicates that the GI

tract provides different niches with the appropriate nutrient, gaseous, and pH conditions that promote certain microorganisms to grow (Table 4.1).

Table 4.1. Enteric bacterial populations in humans. Modified from (2)

	Stomach	**Jejunum**	**Ileum**	**Colon**
Total bacteria/g	$0–10^3$	$0–10^4$	$10^4–10^8$	$10^{10}–10^{12}$
Aerobes and facultative anaerobes/g	$0–10^3$	$0–10^4$	$10^4–10^5$	$10^2–10^9$
Anaerobes/g	0	0	$10^3–10^8$	$10^{10}–10^{12}$
pH	3	6–7	7.5	6.8–7.3

Studies of the composition of the human intestinal microbiota have shown that the intestinal flora is very complex and varies between individuals, but appears to remain constant in a given healthy individual, provided that the flora is not altered with extended antibiotic treatments. (5)

Despite the current dramatic increase in species detected in the gut its diversity is quite low compared to other ecosystems, like soil or marine environments. Of the 70 divisions of the Bacteria kingdom and 13 divisions of the Archeae kingdom

described to date, the human gut is comprised by mainly 2 divisions, Firmicutes and Bacteroidetes.(5) The colon is heavily populated by anaerobes and the bacterial diversity consists of 8 phyla, with members of the gram-negative Bacteroidetes and gram positive Firmicutes constituting 60-80% of the total community.(5) Sixty of the Firmicutes phylotypes belong to the Clostridia class. Surprisingly, some studies did not find rDNA sequences of the Bacteroidetes division, which has been described as a predominant division in other studies. (5) (6) Similarly, genetic fingerprinting studies have failed to isolate members of the Actinobacteria division, including bifidobacteria, which are known to have an important role in gut homeostasis and can be easily detected from fecal and mucosal samples by culture-independent techniques such as FISH and isolated in cultures. (7) This shows the limitations that genetic fingerprinting has, in particular the biases associated with the lysis and DNA extraction protocols, which can miss bacterial population more resistant to lysis. (8)

It has been suggested that possessing the 'right' type of bacteria in the intestine is crucial for intestinal health. Although we are only in the initial stages of understanding the composition of an appropriate intestinal microbial flora, there is a direct link between intestinal microbial dysbiosis and disease, including IBD. A higher concentration of adherent bacteria are present in the intestinal mucosa of patients with intestinal inflammation, and bacterial concentration increases with disease severity, even in uninflammed portions of the gut (9). Absence or low numbers of *Bacteroides* and Firmicutes are a common finding in patients with IBD (9;10). In particular, *Bacteroides* produces a surface polysaccharide (PSA)

which protects against experimental colitis in an animal model via expansion of IL-10 secreting T cells, thus promoting a tolerant state in the gut mucosa (11). Other bacterial groups that have been widely studied for their anti-inflammatory properties are probiotics. Several probiotic strains, including *Lactobacillus sp., Lactobacillus salivarius* and *Bifidobacter infantis* have been shown to prevent or attenuate colitis in IL-$10^{-/-}$ mice (12;13).

The mechanisms of action used by probiotics to reduce inflammation are just beginning to be elucidated. Probiotics and their secreted products can modulate the mucosal immune response mainly through their interactions with intestinal epithelial cells. *Lactobacillus plantarum* prevents colitis in IL-$10^{-/-}$ mice by reducing intestinal permeability and remodeling the structure of the tight junction (14). Similarly, a secreted bioactive peptidic factor from *B. infantis* was shown to attenuate inflammation by normalizing intestinal permeability in IL-$10^{-/-}$ mice (15). Clearly, the interactions between bacteria and the epithelial barrier, whether beneficial or not, play a major role in the establishment of immune homeostasis or inflammation. Our studies have shown that the IL-$10^{-/-}$ mouse has a permeability defect in the small intestine that precedes colitis and that the severity of disease is dependent on the degree of small intestinal leakiness (chapter 3). One of our hypotheses as to how events in the small intestinal epithelium control colitis is that the colonic flora may change as a result of increasing small intestinal permeability. In this chapter I present a summary of the results obtained from assessing the colonic flora of IL-$10^{-/-}$ and wild type mice after increasing their small intestinal permeability.

4.2 Materials and Methods

4.2.1 Animals

Homozygous IL-$10^{-/-}$ mice, generated on a 129 Sv/Ev genetic background, and 129 Sv/Ev wild type mice were housed under specific pathogen-free conditions until weaning (3 weeks), when they were moved to conventional animal housing. The mice were housed in HEPA filter cages and fed a standard mouse chow diet. These mice were bred and raised in the animal facility at the University of Alberta.

4.2.2 Pharmacological therapy

To study the changes in microbial flora of IL-$10^{-/-}$ mice under increased small intestinal permeability conditions, animals aged 3.5 weeks were randomized into 2 groups (n = 9-11 per group). One group of IL-$10^{-/-}$ mice received AT-1002 in drinking water, whereas the other group of IL-$10^{-/-}$ mice received vehicle. The drinking water was prepared daily by dissolving 1 mg/ml of AT-1002 in filtered distilled water. The placebo group drank filtered distilled water as well. Treatment continued until 12 weeks of age, when animals were sacrificed by cervical dislocation.

To assess the effect of AT-1002 on the microbial flora of wild type mice, animals aged 8-9 weeks were randomized into two groups of 5 mice each. Fecal samples were collected from all animals at the beginning of the study. After the initial

collection of fecal pellets, one group received AT-1002 *ad libitum* in drinking water (1 mg/ml) for 4 weeks and the other group received distilled water. At the beginning of each week fecal samples were collected immediately upon defecation, placed in sterile microfuge tubes and flash frozen in dry ice. All samples were stored at -80ºC until analysis.

4.2.3 Denaturing Gradient Gel Electrophoresis (DGGE)

Upon sacrifice the colon was flushed with 10ml of sterile PBS 1X to remove fecal content. One-quarter of the colon (proximal side) was flash frozen and kept at −70°C until DNA extraction. DNA was extracted from the tissue using the Ultraclean Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA), with a few modifications. Tissue samples were incubated for 30 min with 300 µl lysozyme solution (30 mg ml^{-1}) at room temperature, vortexing every 10 minutes. Samples were shaken in a bead-beater for 30 sec at 6000 rpm. Samples were then centrifuged for 30 sec at 10,000 x g and the remaining isolation steps were followed according to the kit instructions. Purified DNA was quantified and frozen at -20°C.

Bacterial DNA was obtained by conventional polymerase chain reaction (PCR) using the universal bacterial primers HDA1-GC and HDA2, which target the V2-V3 region of the 16S rDNA gene, as previously described.(16) The amplified bacterial DNA products were visualized in 2% agarose gel using SYBR Safe (Invitrogen, Carlsbad, CA) and quantified using the ND-1000 spectrophotometer

(NanoDrop, Wilmington, DE). 250 ng of DNA from each sample were loaded onto a denaturing gradient gel and run at 120 V, 60 °C for 240 min. Electrophoresis was performed using a DCode apparatus (Bio-Rad) and 6 % polyacrylamide gels with a 30–55 % gradient of 7.0 M urea and 40 % (v/v) formamide. The running buffer used was TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Gels were stained with SYBR safe for 30 min, washed with distilled water for 15 min, and visualized under UV light. DGGE bacterial profiles were compared using the Bionumerics software (version 6.0, Applied Maths, Austin, TX). The Dice method was chosen to obtain similarity coefficients at a sensitivity of 3%.

4.2.4 Terminal restriction fragment length polymorphism (TRFLP)

DNA for TRFLP analysis was extracted and purified from fecal samples as mentioned in the previous section without the lysozyme treatment. Amplification of bacterial 16S rDNA was performed as previously described (17) with some modifications. Briefly, the V1 and V2 regions of the 16S rDNA bacterial genes were amplified using the 6-FAM labeled 27f (5-GAAGAGTTTGATCATGGCTCAG-3) and 342r (5-CTGCTGCCTCCCGTAG-3). PCR conditions were: one denaturation step of 94ºC for 1 min, 36 cycles of 94ºC x 1 min, 57ºC x 1 min, 72ºC x 2 min and a final extension at 72ºC for 5 min. The PCR amplicons were quantified using the ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) and ~ 150 ng of DNA was digested with the

restriction enzyme HhaI at 37ºC for 4 h. Digestion conditions were: 10 units of HhaI, 10µl of PCR product, buffer C 1X and 20µg of BSA (Promega, Madison, WI). Terminal fragments were separated by capillary electrophoresis on a 3130xl Genetic Analyser (Applied Biosystems, Foster City, CA). 1µl of digested product, 1µl of 500-bp DNA size standard and 28 µl of sample buffer were applied to the capillaries. Traces of the terminal fragments were visualized using GeneMapper Software System (Applied Biosystems, Foster City, CA) and were analyzed with Bionumerics 6.0 software (Applied Maths, Austin, TX). Traces of the samples obtained before AT-1002 treatment (Week 1) and the subsequent 3 samples (Weeks 2, 3 and 4) were compared for each mouse. Only peaks with a relative abundance ≥ 1% were included in the comparison in order to exclude background noise signal. Comparisons were performed based on similarity coefficients using the Dice method, which is based on the presence and absence of peaks and does not consider the area under the peaks.

4.2.5 Enteric bacteria cultures

Colonic contents of 2 wild-type mice, aged 12 weeks, were serially diluted in 1 ml aliquots of sterile tryptic soy liquid medium (BD Biosciences, Sparks, MD). Dilutions were performed from 10^{-1} to 10^{-7} for each sample. A volume of 0.1 ml of each dilution was used to inoculate 9.9 ml tubes containing sterile tryptic soy liquid medium and increasing concentrations of AT-1002 (0, 0.1, 0.5, 1.0 mg/ml).

A set of tubes contained heat inactivated AT-1002 (1.0 mg/ml). Bacterial growth was measured by optical density at 600 nm 16 hrs after the cultures were started.

4.2.6 Statistical Analysis

Normally distributed data are expressed as means ± SEM. Comparisons between groups were made using paired student's t-test (2 groups), or ANOVA (> 2 groups) with a Tukey test for post hoc comparisons. All calculations were performed using Graphpad Prism 3.0 (San Diego, CA), and significance was assumed at $P < 0.05$.

4.3 Results

4.3.1 Increasing small intestinal permeability modifies the colonic adherent flora in IL-10$^{-/-}$ mice

In order to study the effect of increasing small intestinal permeability with AT-1002, total DNA was extracted from colonic tissue of treated IL-10$^{-/-}$ mice and controls, and 16S ribosomal DNA was obtained by PCR. These PCR products were analyzed in DGGE gels in order to compare the band patterns in each sample. Software analysis provided similarity indexes amongst lanes and it showed that band patterns from control and treated animals clustered in different groups based on similarity, suggesting that there was a difference in bacterial population between IL-10$^{-/-}$ mice treated with AT-1002 and placebo (Figure 4.1).

It is important to mention that the tissues chosen for microbial flora analysis were from animals housed in different cages, reducing the possibility that the similarities in bacterial patterns between groups were due to animals sharing the same environment.

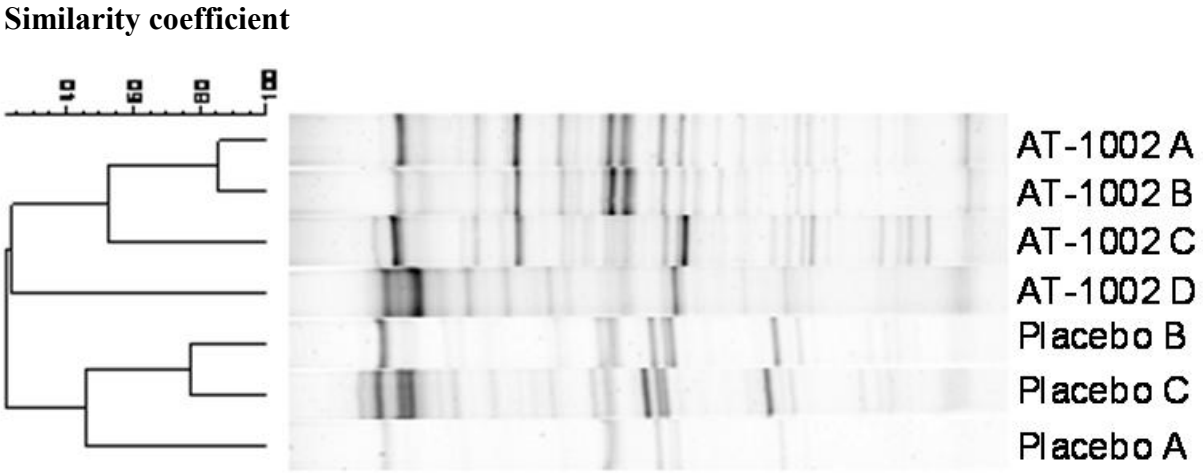

Figure 4.1 AT-1002 changed the microbial flora of AT-1002 treated IL-$10^{-/-}$. DGGE analysis of the colonic adherent bacterial populations of IL-$10^{-/-}$ mice treated with AT-1002 and placebo. Software analysis was used to compare the band patterns between groups and similarity coefficients were calculated. Samples treated with AT-1002 treated or placebo clustered in two distinct groups, indicating that there was an evident change in colonic bacterial flora after AT-1002 treatment, n = 3-4.

4.3.2 Increasing small intestinal permeability modifies the fecal flora in wild type mice

Because the change in microbial flora could be due to the increase in inflammatory response observed in the IL-$10^{-/-}$ mice treated with AT-1002, we tested the effect of AT-1002 on the microbial flora of wild type animals. Briefly, the drug was given to wild type mice in drinking water for 4 weeks and fecal samples were collected once a week. The first sample (week 1) was collected before the beginning of the treatment. 16S rDNA was obtained from the fecal samples by using 2 sets of primers: one set amplified the V2-V3 regions of the genomic bacterial DNA, where as the other set amplified the V1-V2 regions. The PCR products from the former set were analyzed by DGGE and the PCR products from the latter set were subsequently digested and analyzed by TRFLP. In both cases, the band or peak profile from week 1 was compared to the 3 subsequent profiles (weeks 2, 3 and 4).

Software analysis of the band patterns obtained by DGGE and the peak traces obtained by TRFLP produced very similar results. Microbial composition from samples obtained before AT-1002 treatment were similar to samples obtained during weeks 2 and 3, but were significantly different from samples obtained at week 4. Samples obtained from control mice during 4 weeks showed a similar microbial composition (Fig.4.2).

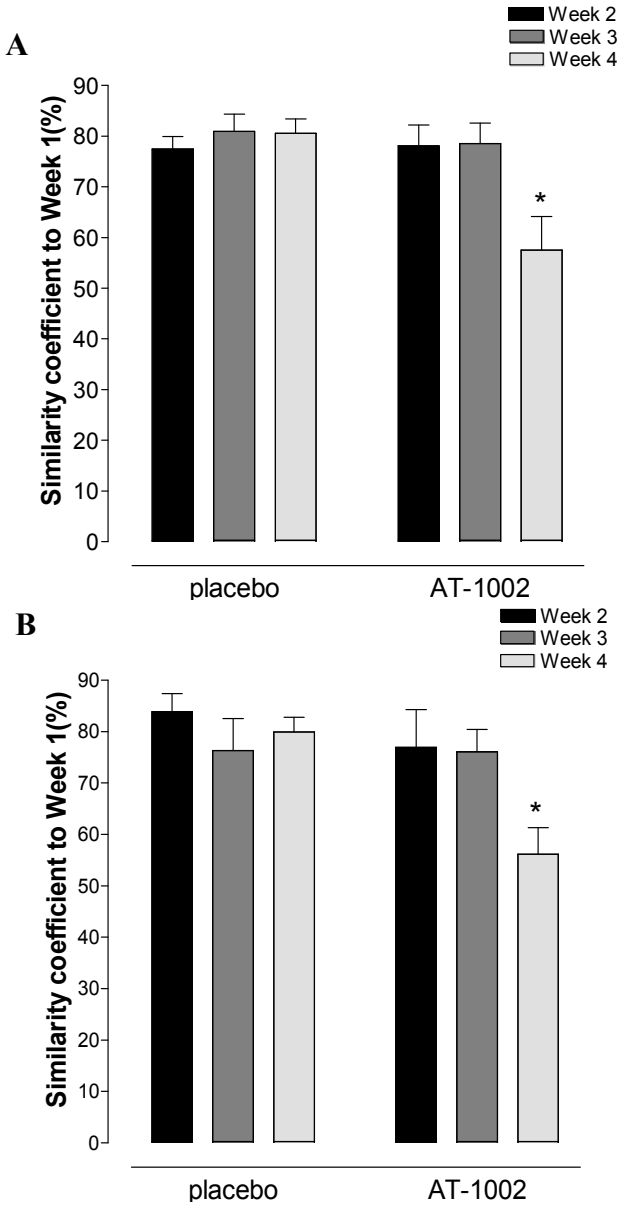

Figure 4.2 Microbial analysis of fecal samples from wild type mice treated with AT-1002 for 4 weeks. Similarity coefficients of DGGE lanes (A) or TRFLP traces (B) were compared between Week 1 and the 3 subsequent weeks. Both techniques showed that by the fourth week of treatment the similarity coefficient of mice treated with AT-1002 decreased significantly, indicating a change in fecal microbial flora; $p>0.05$, $n = 5$.

4.3.3 AT-1002 does not change bacterial growth in vitro

To test whether the observed effect of AT-1002 in microbial flora was a contact effect of the drug with the enteric bacteria, or whether the change in microbial flora was an effect of the change in permeability caused by AT-1002, enteric bacteria were cultured with different concentrations of AT-1002. The drug did not have any effect in the growth of enteric bacteria in liquid culture under aerobic conditions. (Fig. 4.3)

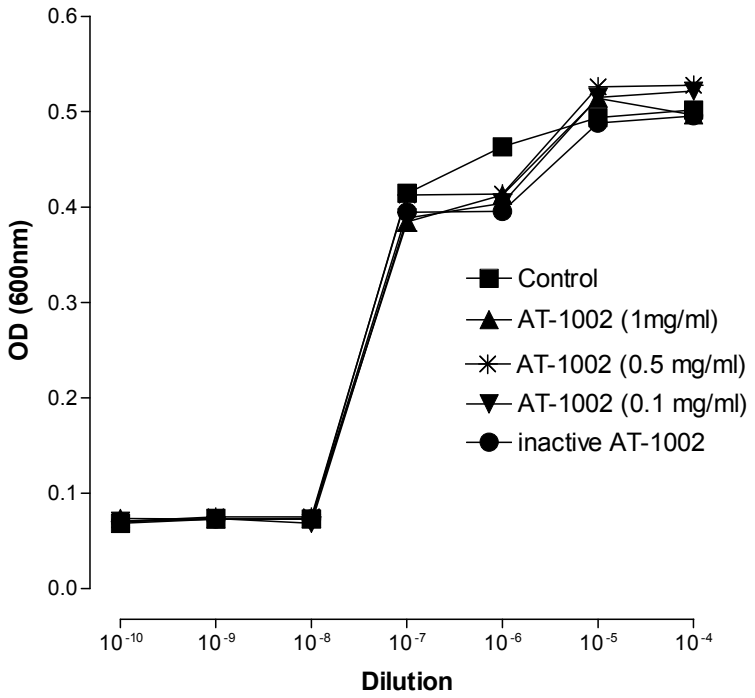

Figure 4.3 AT-1002 does not change enteric bacterial growth *in vitro*. Colonic contents of 2 wildtype mice were serially diluted (10^{-1} to 10^{-7}) in tryptic soy liquid medium. A volume of 0.1 ml of each dilution was used to inoculate tubes containing liquid medium and increasing concentrations of AT-1002 (0, 0.1, 0.5, 1.0 mg/ml) or heat-inactivated AT-1002 (1.0 mg/ml). Optical density measurements at 600 nm performed 16 hours after cultures were started showed that there was no difference in bacterial growth between cultures than contained AT-1002 and controls.

4.4 Discussion

In this chapter we provide the first evidence that increasing small intestinal permeability provokes a change in the adherent colonic microbial flora of IL-$10^{-/-}$ mice and in the fecal flora of wild type mice. We speculate that by increasing small intestinal permeability, material from the lamina propria can leak into the lumen and change the intestinal microbial flora by modifying the environmental conditions that establish the bacterial ecology in the gut. There are many physiological factors that regulate the numbers and types of bacteria than can inhabit the gut. Gastrointestinal secretions like bile and saliva contain substances, like lyzosyme, antimicrobial peptides, and bile acids that limit bacterial growth. The small intestine, in particular, reduces the amount of bacteria through peristaltic movements and the secretion of water and electrolytes that washes microorganisms downstream. (2) Another critical factor is the secretion of IgA, which is present in very high concentrations in the lamina propria (18), therefore it was not surprising to observe that there was an increase in IgA secretion after the 8-week AT-1002 treatment (Figure 3.13). IgA is the main immunoglobulin at mucosal surfaces and it has several key functions in maintaining the immune homeostasis of the gastrointestinal mucosa. By blocking microbial adherence structures, neutralizing toxins and agglutinating microbial cells, IgA readily prevents the entry of both innocuous and potentially harmful microorganisms. (19) Because IgA has such a direct role in shaping the resident microbial flora of the GI tract one could speculate that a long term increase in small intestinal

permeability and increases in IgA secretion could modulate the adherent microbial flora of the colon. DGGE analysis of rDNA bacterial sequences adhered to the colonic mucosa showed that the bacterial population from IL-$10^{-/-}$ animals treated with AT-1002 differed significantly from the one in control animals. However, from these experiments it is unclear whether the increase in IgA was the cause of the shift in microbial population, and if this shift caused the previously described increase in inflammation in the IL-$10^{-/-}$ mice. IgA has a protective role in preventing invasion of microorganisms. However, IgA is normally taken across the enterocytes basolateral surface, actively assembled into sIgA and then secreted by the enterocytes and it is this special comformation that gives it its ability to block and agglutinate cells. It is possible that with increased paracellular permeability there is less IgA available for uptake by the enterocytes and consequently less sIgA secreted. We also considered the possibility that the observed change in bacterial population could be secondary to the increased inflammatory response observed in the AT-1002 treated animals.

We examined the effect of AT-1002 on the fecal flora of wild type mice and showed that in the absence of inflammation there was also a shift in bacterial population. AT-1002 did not have a direct *in vitro* effect on colonic bacterial growth, at least under aerobic conditions. Thus, it is likely that the observed change in intestinal flora was induced by the change in small intestinal permeability. The change in colonic flora could be an important mechanism underlying the observation that an increase in small intestinal permeability increases colonic disease in this animal. However, our experiments do not address

whether the change in colonic flora precedes the increase in inflammation, or that it causes it.

The insensitivity of culturing bacteria from the gut and other terrestrial environments has led to new investigative ways of exploring the biodiversity of these ecosystems. These new techniques detect bacterial small subunit ribosomal RNA genes (16S ribosomal DNA or rDNA). Genetic fingerprinting and sequence analysis of the cloned microbial RNA genes has changed our view of the microbial diversity in the gut and dramatically increased the number of newly described microorganisms. From our experience with these techniques, we obtained very similar results from the analysis of fecal samples from wild type animals using both DGGE and TRFLP, even when the amplicons used in each technique were obtained with different primers. Thus, we can conclude with a good degree of confidence that a shift in fecal microbial population indeed occurred in the animals treated with AT-1002. Initially we proposed to use TRLFP analysis in order to obtain taxonomic information from the samples we analyzed. Unfortunately, an important number of the restriction fragments corresponding to bacterial strains that were different between groups appeared as unknown microorganisms in the restriction fragment databases available to date. Therefore, we could not obtain sufficient taxonomic information to perform comparisons between groups. The use of a more robust technique based on DNA sequencing known as pyrosequencing is recommended for this type of analysis. This technique is currently considered the gold standard for taxonomic analysis of

microbial populations as restriction fragment databases only include a limited amount of species.

To our knowledge, the work presented in this chapter shows for the first time that increasing small intestinal permeability for an extended period of time (at least 4 weeks) has a measurable effect in the composition of the microbial flora in wild-type and IL-$10^{-/-}$ and that it might be a mechanism by which small intestinal permeability modulates disease severity in this mouse model.

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Chapter 5

Effect of increasing small intestinal permeability in the development of oral tolerance

5.1 Introduction

As discussed in previous chapters, IBD is a multifactorial disease that occurs due to an aberrant immune response to the commensal intestinal microbiota. Genetic associations have been identified in human IBD patients, involving the genes NOD2/CARD15 (1), OCTN1/2 (2), ATG16L (3) and IL-23R (4). Not surprisingly, most of these genes are involved in immune functions. The immune system associated with the intestinal mucosa is referred to as the gastrointestinal associated lymphoid tissue (GALT). Strategically placed in areas where external pathogens and antigens may gain access to the body, the GALT must integrate complex interactions among diet, external pathogens, and local immunological and non-immunological processes. It has been estimated that approximately 25 % of the intestinal mucosa is made up of lymphoid tissue (5). The GALT is composed of aggregated tissue in the form of Peyer's patches (PP) and solitary lymphoid follicles, and non-aggregated cells in the lamina propria and intraepithelial regions of the intestine, as well as mesenteric lymph nodes (MLN) (6) (Figure 5.1).

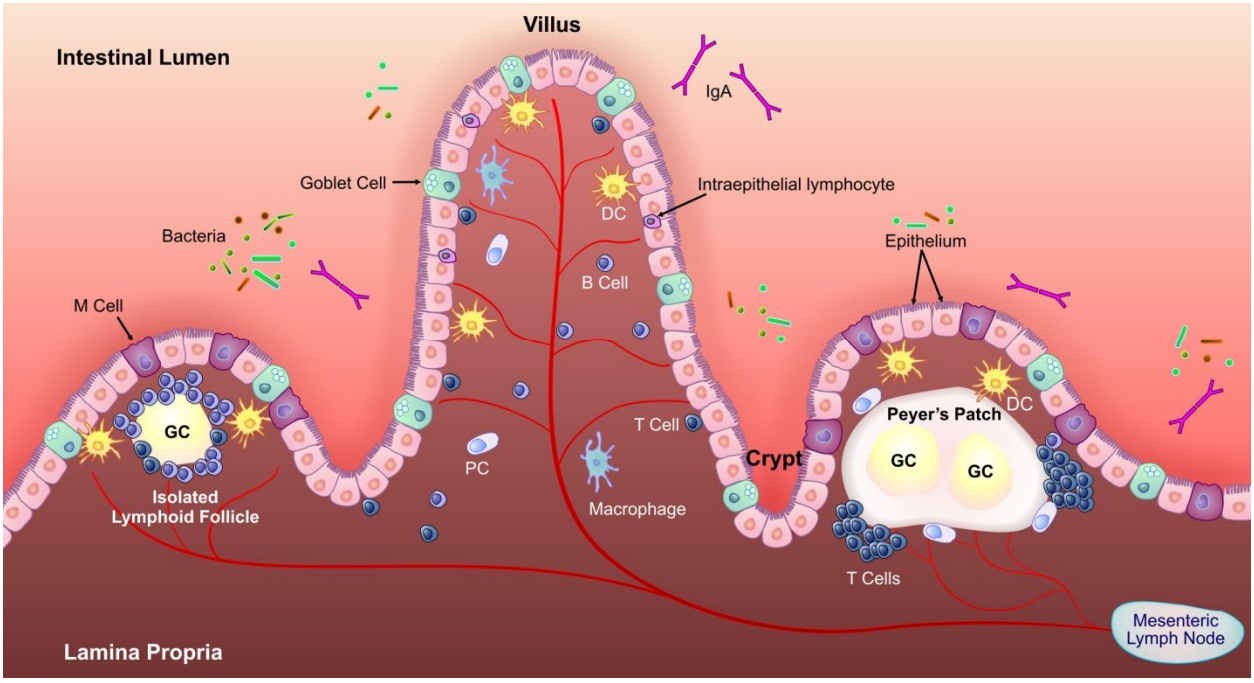

Figure 5.1. Diagram of the gut-associated lymphoid tissue (GALT). The GALT consists of aggregated lymphoid follicles (PP and isolated lymphoid follicles) and the diffuse or non-aggregated tissue (lamina propria). The epithelium of aggregated follicles contains M cells, which are specialized in antigen sampling. Underneath is the subepithelial dome, which consists of germinal centers (GCs) of B cells in different stages of maturation, as well as dendritic cells (DCs) and T cells. Aggregated follicles are the inductive sites of immunity, where naïve T and B cells are activated, to then migrate to the (MLN) and into the effector sites of the GALT, the lamina propria. Within the lamina propria, mature B cells or plasma cells (PC) produce IgA, which gets actively transported into the intestinal lumen (7).

PPs are aggregates of lymphoid follicles found throughout the mucosa and submucosa of the small intestine. These patches contain both CD4+ and CD8+ T-cells, as well as naïve B-cells, plasma cells, macrophages and dendritic cells (DC) (6). Overlying the PPs are specialised epithelial cells known as M-cells, which endocytose, transport and release antigens from the gut into the PPs, where these antigens are presented on antigen presenting cells (APC) to T- and B-cells (6;8). Upon activation, B-cells undergo class-switching to produce IgA antibodies, a process that is facilitated by both activated CD4+ and CD8+ cells (6;8). Activated immune cells exit the PPs via the MLNs, enter the systemic circulation by way of the thoracic duct, and then specifically home back to populate the lamina propria and intraepithelial regions of the intestine (6;8;9). There is some evidence that suggests that there is even a certain preference of activated immune cells to migrate to effector sites in the vicinity of the effector site where the initial activation occurred (10). This suggests that the mucosal immune system compartmentalizes the cell-mediated immune response minimizing the involvement of the systemic immune system, unless it is required.

PPs represent a major ‘sampling’ site for particulate intestinal antigens. Solitary lymphoid follicles are present throughout the length of the intestinal tract, particularly in the colon and rectum, and have M-cells associated with the overlying epithelium (11). At present it is assumed that these follicles are functional equivalents of PPs.

The lamina propria consists of a diffuse population of T- and B-cells, plasma cells (PC), mast cells and macrophages, all covered by a single layer of epithelial cells (6). The role of the intestinal epithelial cells (IECs) or enterocytes has evolved from cells involved mainly in digestion, absorption and transport of nutrients, to also a main role in immune regulation of the underlying cells of the lamina propria.

IECs are known to produce different chemokines and cytokines depending on the type of microbial molecules that become in contact with the epithelium (12). IECs have been shown to condition the immune response of dendritic cells by inducing the secretion of tolerogenic signals (IL-10) over active immunity signals (IL-12) (13). The enterocyte is also involved in antigen transport to underlying APCs, or they can present antigen themselves to lymphocytes. Moreover, the enterocyte is polarized in such way that antigen is processed within the apical portion of the cytoplasm and can be presented in the context of MHC class II in the basolateral surface (14).

Although not situated within the intestinal mucosa, the MLNs are considered part of the GALT. MLNs are composed of immune cells leaving and entering the gut and those that are part of the peripheral circulation. Immune cells drain to the intestinal lymphatics after differentiation in PPs, and pass through MLNs en route to the thoracic duct and then again en route back to the lamina propria regions of the gut (15).

It is critical that protective immune responses are made to potential pathogens, yet it is equally important that hypersensitivity reactions to dietary antigens are minimized. Thus, most immune reactions to commensal bacteria and dietary antigens provoke a state of hyporesponsiveness towards those antigens, in a process known as oral tolerance. Oral tolerance is considered to be a fundamental component of immunological tolerance – the mechanism by which the immune system discriminates between self and non-self. Central tolerance accounts for the unresponsiveness to self antigens induced in central lymphoid organs like the thymus and the bone marrow (16), where as peripheral tolerance regulates peripheric self-reactive lymphocytes that were not selected against during the initial sorting (17). Unlike central and peripheral tolerance to self antigens, oral tolerance prevents immunological responses to 'safe' foreign antigens. However, many of the mechanisms that the immune system uses to achieve peripheral tolerance also exist for mucosal tolerance. These mechanisms include clonal anergy, clonal deletion, and active suppression (18). However, unlike systemic peripheral tolerance, mucosal tolerance to a foreign antigen also involves a strong and specific humoral response to that antigen by IgA-producing plasma cells (19). This dichotomy between cell-mediated hyporesponsiveness and humoral immunity represents, like any other physiological response, a necessary evolutionary adaptation to the unique conditions in the mucosae, which are absent in the thymus or parenchymal tissues. Also, this 'half-tolerance' towards an innocuous foreign antigen suggests that at least some mechanisms underlying this response may not be the same as the ones in systemic tolerance. Most of the

lymphocytes in the LP are $CD4^+$ T cells that produce particular cytokines (mostly IL-4, IL-10 and TGF-β) that induce B cell differentiation into IgA producing plasma cells and actively suppress cell-mediated immunity (19). B cells in the effector sites are mostly plasma cells that produce IgA. This immunoglobulin is massively produced in the gut mucosa (~5g per day!) and it represents one of the first lines of defense against adhesion of bacteria to the mucosa, as well as neutralization of toxins and prevention of antigen internalization into the body (20). Most of the IgA is secreted into the gut lumen, and takes the form of secretory IgA, distinct from serum IgA. Secretory IgA (sIgA) is a dimer of two monomeric IgA molecules with an attached secretory component (the cleaved extracellular domain of a transmembrane protein expressed on intestinal epithelial cells). The secretory component facilitates the transport of IgA through the epithelium and into the gut lumen and protects IgA from degradation by intestinal enzymes and toxins (11).

In general, the main function of the GALT is to discern between potentially harmful and innocuous antigens while maintaining mucosal and systemic homeostasis. In the former case the physiological response involves humoral and cell mediated immunity in the mucosal tissue and systemically. In the latter case it favours a mucosal humoral response in the absence of systemic cell-mediated immunity or delayed-type hypersensitivity.

The induction of mucosal tolerance depends on a number of factors, including the nature of the antigen, the dose of the antigen, the site of antigen uptake or entry,

the context of antigen presentation, the genetic background, the age of the host and the indigenous flora (21).

In terms of the type of antigen, it has been shown that the uptake of particulate antigens and T-cell dependent antigens regularly favours immunity vs. tolerance. In contrast, soluble proteins tend to induce tolerance. (22). The site of antigen uptake is somewhat dependent on the nature of the antigen and it is critically important for the outcome of the immune response. Antigen can reach the GALT in three ways. The first and most described way is through M cells as explained before, and particulate antigens use this route preferentially, where as tolerogen antigens are less likely to use this route (23). Even though particulate antigens are usually not tolerogens, it has been shown that $CD4^+$ T cells in the PP secrete TGF-β, so this may be one pathway used to induce tolerance. The second route is the transcellular route and it involves the processing of the antigens in IECs. IECs have been also shown to secrete certain cytokines *in vitro* that are known to downregulate a Th1 response and to allow differentiation of lamina propria B cells into IgA-producing plasma cells (24). The third route is through the dentrite extensions of dendritic cells (DCs) into the gut lumen (25). The paracellular route, via the junctional complex, is not considered a normal route of antigen entry as in healthy states it is generally impermeable to most large molecules. However, it has been shown that when permeability is compromised by inflammation, which would enable entry through paracellular route, oral tolerance is jeopardized (26;27).

In this chapter we discuss another possible mechanism by which small intestinal permeability may control colonic inflammation in the IL-$10^{-/-}$ mouse. We hypothesized that by breaching the small intestinal epithelium there would be an increased entry of luminal dietary and bacterial antigens that could alter the physiological mechanisms of antigen uptake and/or presentation, leading to a loss of oral tolerance to these antigens. We present in this chapter a proof-of-concept study in which a normal host was orally exposed for the first time to ovalbumin (OVA) under increased small intestinal permeability conditions and compared it to the tolerant response induced when OVA was given alone.

5.2 Materials and Methods

5.2.1 Animals

Male 129 Sv/Ev mice were housed under specific pathogen-free conditions until weaning (3.5 weeks), when they were moved to conventional animal housing. The mice were housed in HEPA filter cages and fed a standard mouse chow diet. These mice were bred and raised in the animal facility at the University of Alberta. All animal procedures were conducted in accordance with protocols approved by institutional Animal Care and Use Committees.

5.2.2 Feeding and immunization regime

Three groups of 8-10 week-old wild type mice were orally gavaged once daily for five consecutive days with: 20 mg OVA (Sigma, St. Louis, MO), 20 BSA, or 20 mg OVA + 6 mg AT-1002. The group that received AT-1002 by gavage also received the drug *ad libitum* in drinking water (1 mg/ml), whereas the other two groups received regular water. Two days after the last gavage all animals were immunized subcutaneously with 50 µg of OVA + complete Freunds adjuvant containing 50µg *Mycobacterium tuberculosis* (Difco, Detroit, MI). Two weeks later animals were gavaged once daily for 2 days with either 20mg of OVA or BSA. The next day the animals were sacrificed by cervical dislocation. (Fig. 5.2)

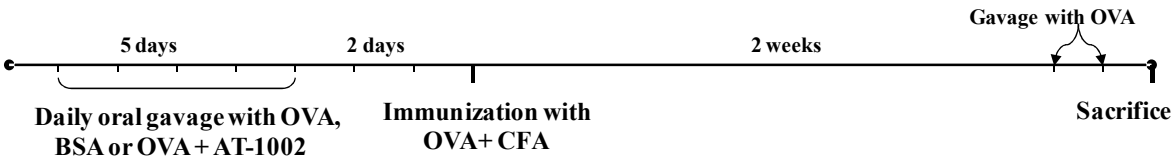

Figure 5.2 Feeding and immunization regime.

5.2.3 Cell cultures and proliferation assay

Spleens and MLN were dissected from the mice, minced between 2 frosted glass slides and strained through a 40 µm mesh strainer. Cells were harvested in complete RPMI-1640 with 10% v/v fecal bovine serum (FBS) and depleted of red

blood cells by osmotic shock with distilled water. MLN cells were pooled within each group. Cell numbers were calculated with the Z2 particle count and size analyzer (Beckman Coulter, Brea, CA). Splenic and MLN cells were placed into the wells of 96 well plates at a concentration of 2 X 10^5 cells per well. Cells were stimulated with 100µg/ml OVA, plate-bound anti-CD3e clone 145-2C11 as a stimulation control (PharMingen, Canada) or medium alone as a negative control for 72 h at 37°C in a humidified incubator at 5% CO2. After 48 h of incubation, the plates were centrifuged and 150 µl supernatants were removed and stored for cytokine assays. Culture media was replaced with media containing [^{3}H] thymidine (1µCi/well) and plates were incubated for another 24 h. Cells were harvested and incorporation of thymidine was measured using the 1450 LCS Microbeta Trilux scintillation counter (Pelkin Elmer, Maltham, MA).

5.2.4 Flow cytometry

OVA-induced cell population changes were assessed by incubating 1 X 10^6 spleen cells in 12 well plates with complete RPMI-1640 with 10% v/v fecal bovine serum (FBS). Cells were stimulated with 100µg/ml OVA or 100µg/ml BSA as a negative control. After 72 h of incubation cells were washed and stained with the following fluorescence-coupled monoclonal antibodies (mAbs): anti-CD11b-phycoerythrin (PE), anti-CD4-PE, anti CD25-fluorescein isothiocyanate (FITC), anti CD3e-PerCP-Cy5.5, anti CD19- PE, anti IL-10-FITC, anti CD11c-FITC, anti CD8-PerCP-Cy5.5, anti CD25-allophycocyanin (APC) and anti FoxP3-PerCP-

Cy5.5. All antibodies were purchased from eBiosciences (San Diego, CA). Cells were washed twice with PBS-1% BSA-0.01% sodium azide (NaN_3), then fixed in 1% paraformaldehyde and analyzed in a FACSCalibur flow cytometer (BD, San Jose, CA).

5.2.5 Analysis of anti-OVA Igs

Levels of OVA-specific Ig isotypes were determined by ELISA as previously described (28) in splenocyte culture supernatants (IgG) or intestinal tissue culture supernatants (IgE and IgA). Briefly, ELISA plates (Nunc, Rochester, NY) were coated overnight at 4ºC with 2µg/ml of OVA in 50mM sodium carbonate buffer with 0.02% NaN_3 (pH 9.6). Plates were washed with PBS with 0.05% Tween-20 and blocked for 2 h with PBS with 0.25% casein. Supernatant samples were serially diluted from 1:1-1:100 (spleen supernatants) or 1:5-1:1000 (gut tissue supernantants) in PBS with 0.25% casein and each dilution was plated and incubated for 2 h at room temperature. Plates were washed 3 times and incubated with biotinylated goat anti-mouse IgG (1:1000), IgA (1:50000) or IgE (1:6000) for 1h. Plates were washed 5 times and incubated with streptavidin- HRP (1:200). Plates were washed 3 times and incubated in the dark for 10-20 min with substrate solution containing H_2O_2 and tetramethylbenzidine. H_2SO_4 (2N) was used as a reaction stop solution. All antibodies and ELISA solutions were purchased from R&D Systems (Minneapolis, MN). Optical densities were determined at 450nm

using a Victor^2 Wallac ELISA plate reader (Waltham, MA). Ig levels were calculated by adding up the optical densities for all dilutions per sample. This type of quantitative analysis has been shown to be accepted in the absence of external standards and is more reliable than single point values at a single dilution (29;30).

5.2.6 In vitro cytokine secretion by intestinal tissue

Upon dissection, whole colons and 8 cm sections of the proximal jejunum were removed, flushed with PBS, cut longitudinally and resuspended in tissue culture plates (Falcon 3046; Becton Dickinson Labware, Lincoln Park, NJ) in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml) and a protease inhibitor cocktail containing AEBSF (104 μM), Aprotinin (0.085 μM), Bestatin (4 μM), E-64 (1.4 μM), Leupeptin (2 μM), Pepstain A (1.5 μM; Sigma, St. Louis, MO) Cultures were incubated at 37°C in 5% CO_2. After 24 h, supernatants and tissues were homogenized and stored at −70°C for analysis of cytokine and protein levels. Cytokine production was measured by ELISA using the following antibodies: anti-IFN-γ, anti-IL-2, anti-IL-10 and anti-TGF-β. All antibodies, cytokine standards and reagents were purchased in kits (DuoSet, R&D Systems, Minneapolis, MN, USA). Cytokine concentrations were normalized per amount of protein in each sample. Protein determination was performed with the BioRad DC Protein assay kit (Hercules, CA).

5.2.7 Statistical analysis

Normally distributed data are expressed as means ± SEM. Comparisons between groups were made using paired student's t-test (2 groups), or ANOVA (> 2 groups) with a Tukey test for post hoc comparisons. All calculations were performed using Graphpad Prism 3.0 (San Diego, CA), and significance was assumed at $P < 0.05$.

5.3 Results

5.3.1 AT-1002 prevented oral tolerance to OVA

To determine if increasing small intestinal permeability would have an effect in the development of oral tolerance towards an antigen, mice were fed OVA, OVA with AT-1002 or the control protein BSA. Two days later they were immunized systemically with the antigen and 2 weeks later the immune response towards OVA was measured in the spleen and MLNs. Proliferation assays of spleen cells showed that when animals were fed OVA, compared to the BSA-fed controls, their splenocytes did not proliferate in the presence of OVA *in vitro,* suggestive of the development of tolerance (Figure 5.3a). When animals received OVA with AT-1002 their splenocytes proliferated, similar to cells from the BSA-fed mice, suggesting that tolerance to OVA has not been established (Figure 5.3a). Surprisingly, when the same assay was performed with cells isolated from MLNs, there was no difference in the rate of proliferation to OVA among treatments (Figure 5.3b).

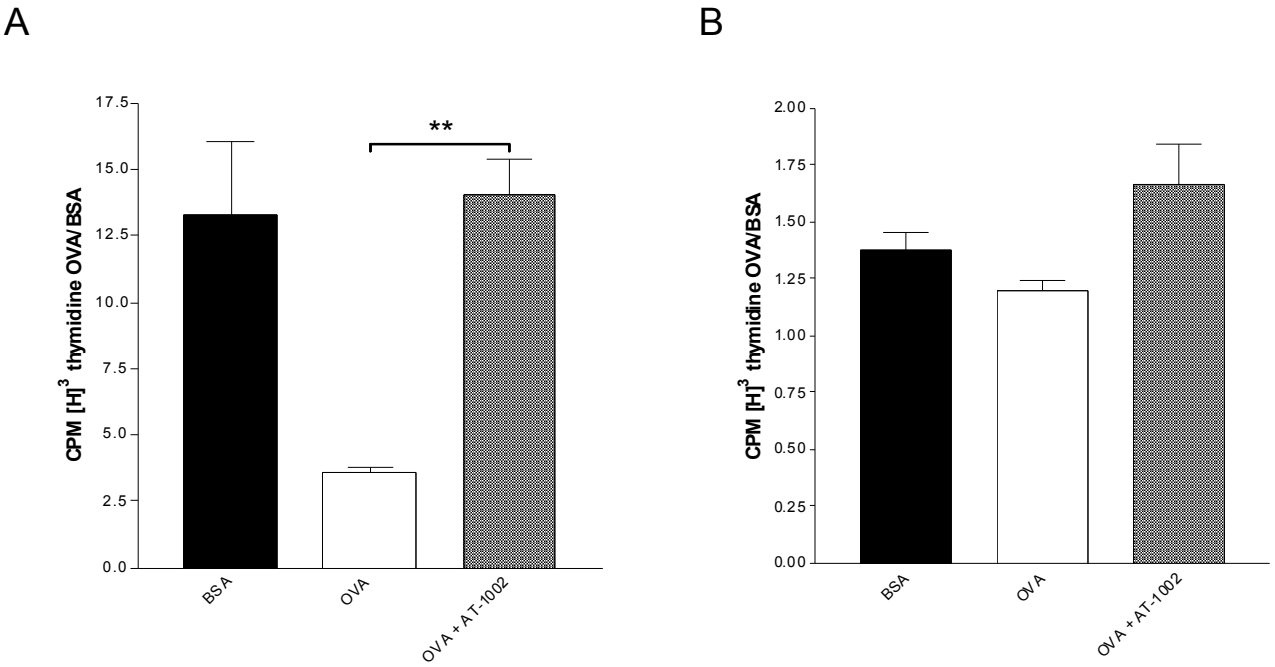

Figure 5.3 Proliferation assay of spleen and MLN cells to OVA. Wild type mice were fed OVA, or OVA + AT-1002 for 5 days. Control animals received BSA. Mice were later immunized with OVA and adjuvant and the spleen and MLN cells were harvested 14 days later. Proliferation reactions were measured by [^{3}H] thymidine uptake of cells cultured with OVA or BSA as a negative control, in triplicate wells. Bars represent the ratio of [^{3}H] thymidine uptake in wells stimulated with OVA to wells cultured with BSA. There was a significant increase in splenocyte proliferation in response to OVA (a), whereas MLN cells did not show a difference in proliferation between groups (b). n=5-8; ** $p<0.05$.

To characterize the proliferative response observed in the spleen, cells incubated with and without OVA for 72 h were stained with different mAbs so as to establish the cell type(s) involved in the cell expansion process. Cells were stained with anti CD3e, a marker of T cells and anti CD19, a marker of B cells and the proportion of cells staining positive for each mAb determined using flow cytometry. After stimulating the cells *in vitro* with OVA it was observed CD19 positive cells or B cells showed an increase in proportion of cells, where as CD3 positive cells decreased in proportion to the splenocyte population (Figure 5.4). Similar to what the proliferation assay showed, CD19 positive spleen cells from animals fed OVA + AT-1002 and animals fed BSA proliferated significantly more than CD19 positive spleen cells from animals fed OVA alone (Figure 5.4). There was no significant stimulation of cells with the negative control protein (data not shown).

Spleen cells were also stained with antibodies against CD4, CD8, CD25, FoxP3, IL-10, IL-4, IL-5, CD11b and CD11c. There was no significant change in CD4, CD8, IL-4 or IL-5 positive cells in any of the groups (data not shown). However, there were significant, yet small changes in the proportion of regulatory T cells (CD4+/CD25+/IL-10+/FoxP3+), CD11b+ and CD11c+ spleen cells. For these 3 cell types, there was significant increase in population from animals fed OVA + AT-1002 compared to the BSA fed group (Figure 5.5).

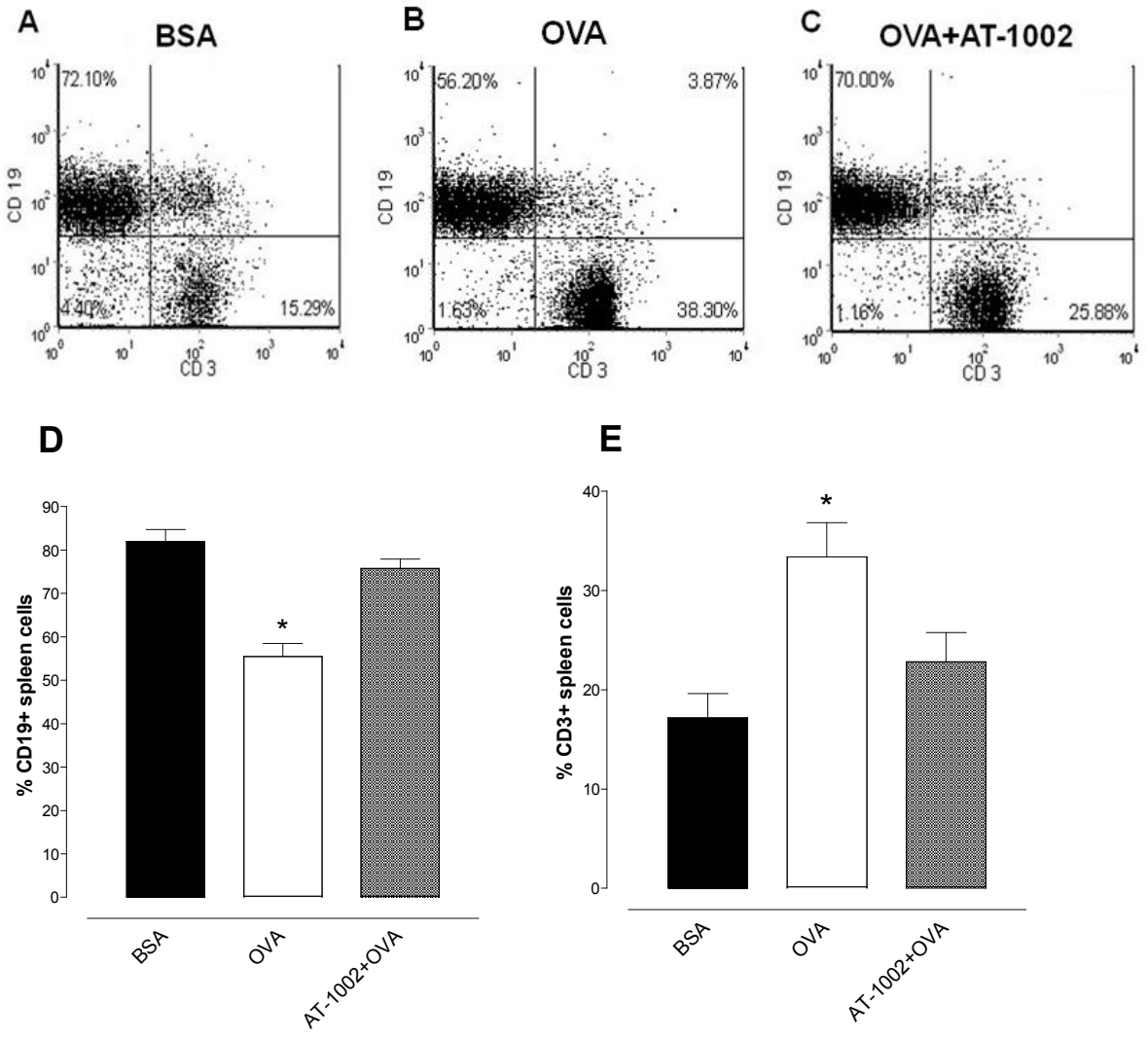

Figure 5.4 Spleen cell population change induced by OVA. Splenocytes from animals fed BSA (a), OVA (b), or OVA + AT-1002 (c), were cultured in triplicate wells in the presence of OVA for 72 h. Cells were stained with fluorescent anti-CD3, a marker of T cells, or anti-CD19, a marker for B cells. A significant decrease in the proportion of CD19 positive cells (D) and an increase in CD3 positive cells (E) was observed in the spleen cells from animals that were fed OVA, compared to animals fed BSA or OVA+ AT-1002. Bars represent the percentage cells after stimulation with OVA. n=5-8; * $p<0.05$.

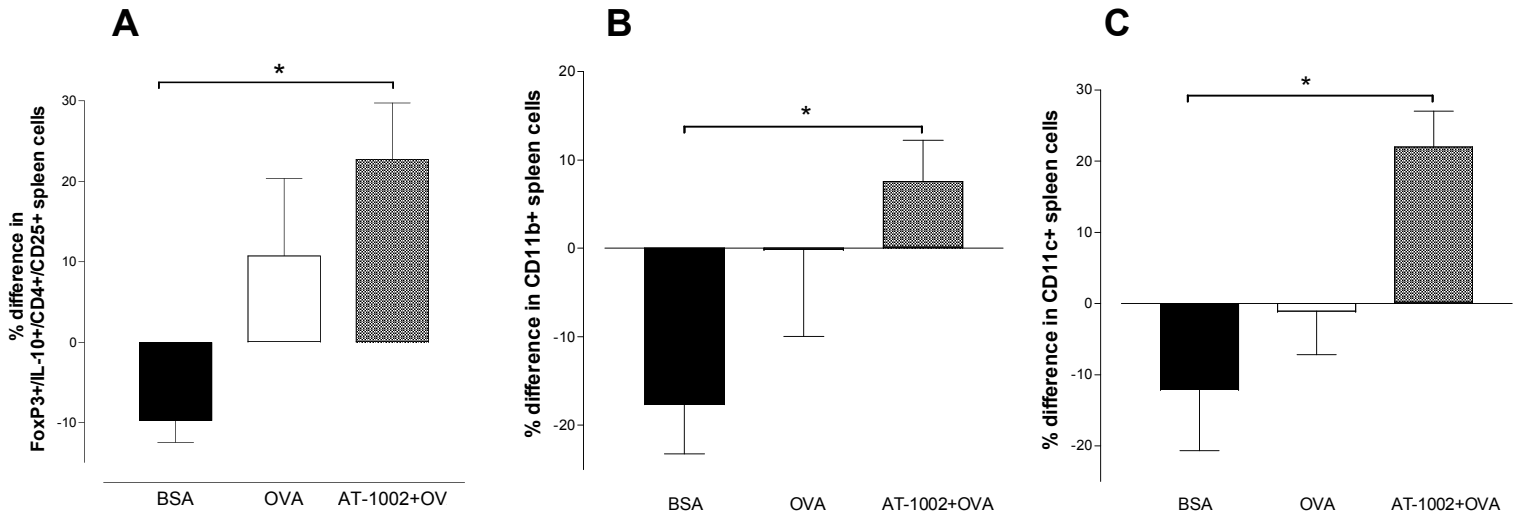

Figure 5.5 Proliferation of other cell types in the presence of OVA. Splenocytes from animals fed BSA, OVA, or OVA + AT-1002 were cultured in triplicate wells in the presence of BSA or OVA for 72 h. Cells were stained with fluorescent anti-CD4/CD25/FoxP3/IL-10 (a), anti-CD11b (b), or CD11c (c) and fluorescence was measured by flow cytometry. A significant increase in all markers was observed in splenocytes from animals fed OVA + AT-1002, compared to animals that were fed BSA. Bars represent the percentage difference in fluorescence between the wells cultured with OVA and wells cultured with negative control. n=5-8; * $p<0.05$

5.3.2 AT-1002 increased the humoral response to OVA.

Because there was an increase in the proportion of B cells when exposed to OVA *in vitro* we determined if the same changes could be observed functionally by measuring the humoral response in the spleen (IgG) and in the gut mucosa (IgA and IgE). Similar to what was observed in the B cell response there was a significant increase in OVA-specific IgG production in the splenocyte culture supernatant from animals fed OVA + AT-1002 compared to animals that ingested OVA or BSA (Figure 5.6a). Consistent with the *ex-vivo* splenocytes experiment, a similar result was observed for OVA-specific IgA in the gut when comparing the OVA and the OVA+AT-1002 (Figure 5.6b). The amount of IgA in the mucosa of BSA treated mice did not differ from the OVA+AT-1002 treatment. There were no changes in IgE secretion among the 3 treatment groups (Figure 5.6c).

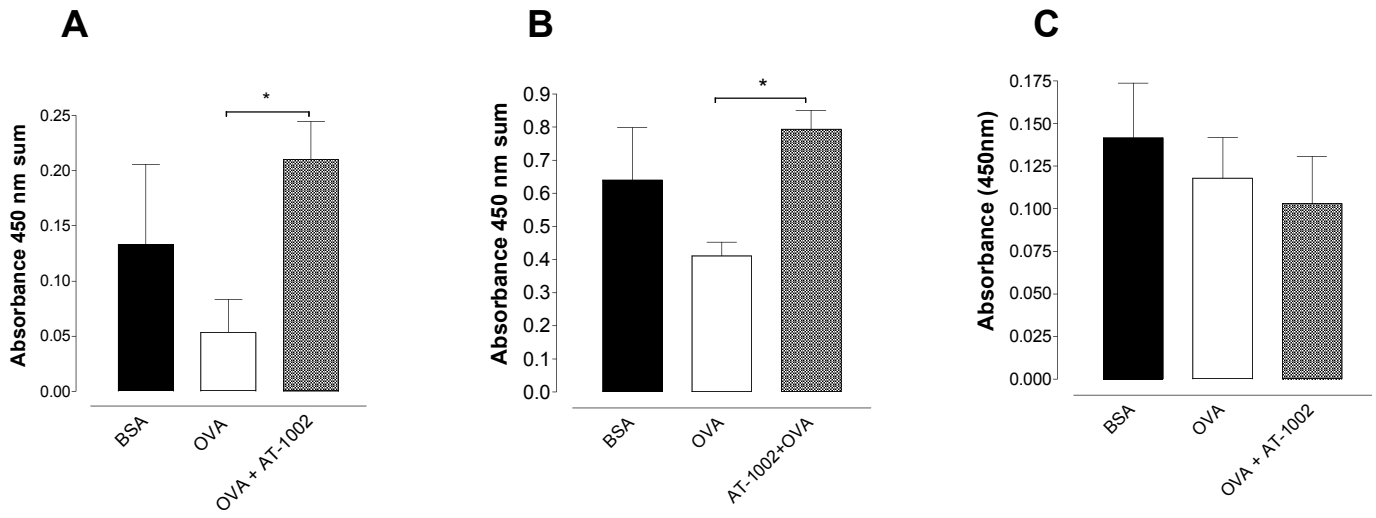

Figure 5.6 OVA-specific antibody secretion. Specific anti-OVA IgG (a), IgA (b), or IgE (c) was measured by ELISA in the supernatant of splenocytes cultured with OVA (a) or the supernatant of small intestinal tissue explants cultured with OVA (b,c). There was a significant increase in OVA specific splenic IgG and small intestinal IgA in animals fed OVA + AT-1002, compared to animals fed OVA alone. n=5-8; * $p<0.05$

5.3.3 AT-1002 changed the intestinal immune response to OVA

In order to study the immune response towards OVA in the gut we measured the cytokine response to OVA exposure in tissue explant cultures of small intestine and colon. Tissues were cultured in cell culture media, proteinase inhibitors and OVA for 24 h. The tissues and supernatants were homogenized and ELISA assays were performed to measure the Th1 type cytokines IFN-γ and IL-2, as well as the cytokines involved in oral tolerance, IL-10 and TGF-β. .

There were no significant differences in IFN-γ and IL-2 production in the small intestines after OVA treatment amongst the three groups (Figure 5.7a,b). However, there was a significant increase in IL-10 and TGF-β concentrations in the small intestines from animals that were orally exposed to OVA alone. This cytokine response towards a fed antigen was prevented when OVA was given with AT-1002 (Figure 5.7c,d).

The cytokine response was also measured in colonic tissue explants. There were no significant differences in IFN-γ or TGF-β concentration amongst treatments after OVA exposure (Figure 5.8a,d). However there was a significantly lower IL-2 concentration in the groups fed with OVA and OVA + AT-1002 compared to the BSA treatment. This suggests that feeding OVA in either the absence or presence of AT-1002 did not induce a proliferative response (as indicated by IL-2) to this antigen in the colon (Figure 5.8b). Most interestingly, colonic IL-10 concentrations were higher in response to OVA compared to BSA and this did not occur in colons from OVA + AT-1002 fed mice (Figure 5.8c), suggesting that

even when the permeability change occurred in the small intestine this immune response towards OVA was observed in the colon as well.

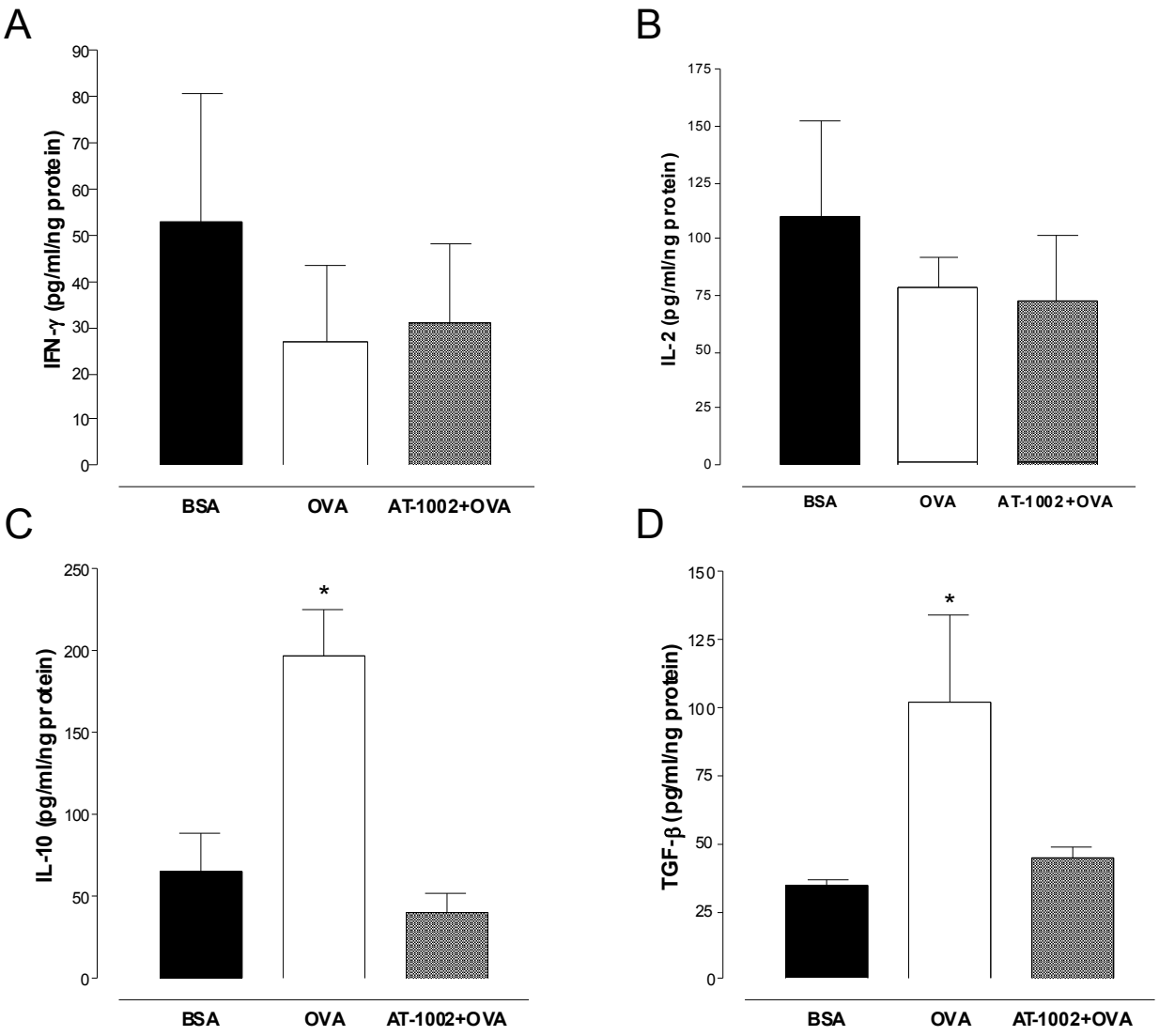

Figure 5.7 Cytokine production in small intestinal tissue explants. Production of IFN-γ (a), IL-2 (b), IL-10 (c) and TGF-β (d) was measured by ELISA in the supernatant of small intestinal tissue explants from animals fed OVA, OVA + AT-1002 or BSA. Bars represent the average of triplicate wells cultured with OVA for 24h. Administration of AT-1002 with OVA prevented the increase in IL-10 and TGF-β produced in orally tolerised mice. n=5-8; * $p<0.05$

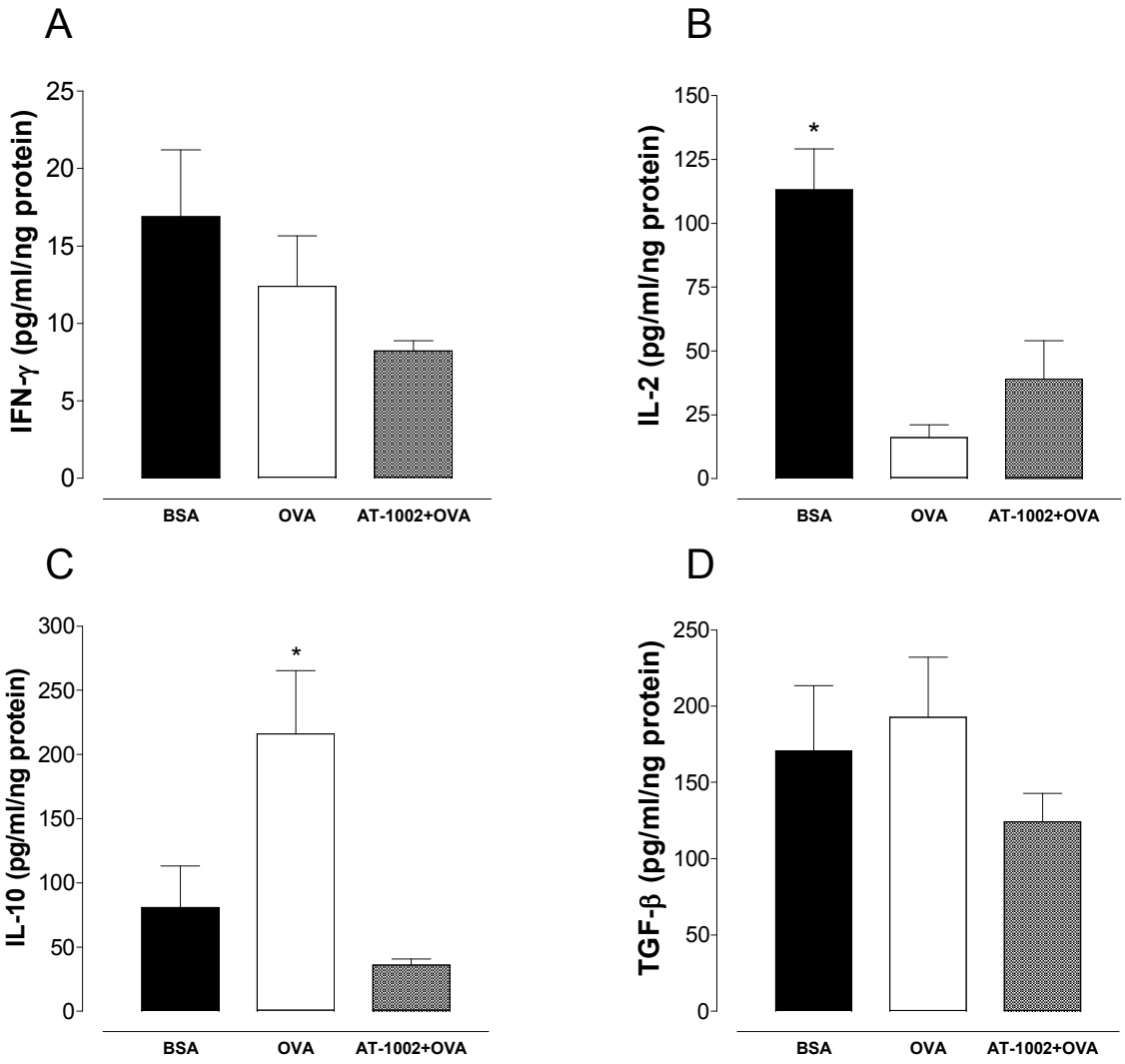

Figure 5.8 Cytokine production in colonic tissue explants. Production of IFN-γ (a), IL-2 (b), IL-10 (c) and TGF-β (d) was measured by ELISA in the supernatant of colonic tissue explants from animals fed OVA, OVA + AT-1002 or BSA. Bars represent the average of triplicate wells cultured with OVA for 24h. There was a significant reduction in IL-2 production in explants from animals fed OVA or OVA + AT-1002. In addition, administration of AT-1002 with OVA prevented the increase in IL-10 produced in orally tolerised mice. n=5-8; * $p<0.05$

5.4 Discussion

The intestinal immune system has the difficult task of discriminating between pathogenic organisms and innocuous dietary or commensal bacterial antigens. This constant decision-making process takes place in an environment with the highest antigenic load in the body. As a result, the physiological adaptation to such conditions is a status of immune hyporesponsiveness to non harmful antigens, or oral tolerance. This tolerant state is achieved by actively suppressing cell mediated immunity and promoting B cell differentiation into IgA-producing plasma cells in an environment rich in IL-4, IL-10 and TGF- β (19). This adaptation prevents potentially harmful cell mediated immune responses that would injure the mucosa. An intact mucosal architecture is important in the induction of oral tolerance because it controls for proper antigen uptake and presentation. The small intestinal mucosa possesses the majority of lymphoid structures and it is likely the major site where oral tolerance is induced (18). Therefore, we believe that a breach in the small intestinal mucosa can lead to a breakdown of the processes involved in oral tolerance induction and to inflammatory diseases like IBD. In this study we showed that increasing small intestinal permeability has a significant effect in the development of oral tolerance in a normal host. Performing this study in wild type animals was important in order to determine whether an increase in paracellular permeability would compromise oral tolerance in the absence of inflammation,.

In a normal host the immune response to an oral antigen is characterized by the production of specific IgA in the intestinal mucosa and the absence of a systemic

immune response towards the fed antigen. In this study we showed how simply increasing small intestinal paracellular permeability induced a systemic immune response to OVA. This immune response was characterized by OVA-specific antibodies producing B cells, suggesting that increasing small intestinal permeability alone is not sufficient to induce a T cell-mediated immune reaction in a healthy host. In a host genetically predisposed to inflammation, such as the IL-10$^{-/-}$ mouse, the immune response to an antigen under increased small intestinal conditions may be one of cell-mediated immunity instead of a humoral response, leading to a chronic inflammatory state and tissue injury. This may be the same in IBD patients as they have been shown to fail to develop oral tolerance to dietary antigens (31) as well as their own commensal bacterial flora (32;33) and they also have higher titers of antibodies against food antigens in blood (34).

When OVA was fed with AT-1002, there were also changes in the expansion of other cell populations: CD4+/CD25+/FoxP3+ regulatory T cells, CD11b+ cells and CD11c+ cells. In contrast to the observed expansion of B cells, the increase in those cell populations was exclusive to the group of animals fed with AT-1002 + OVA. This suggests that although there was a similar B cell expansion in both the AT-1002 + OVA fed group and the BSA fed control group, there are other immune mechanisms that are triggered when small intestinal permeability is increased. Regulatory T cells are an important group of cells in the downregulation of Th-1 type immune responses. Perhaps the expansion of regulatory T cells in the animals that received AT-1002 is a necessary mechanism induced in a normal host in order to avoid a Th-1 type response towards an

antigen that does not enter the gut mucosa via the classical routes of antigen entry. Similarly, the relative increase in CD11b+ and CD11c+, markers of macrophages and dendritic cells respectively, may occur as a response to an increase in intestinal permeability and the consequent increase in antigen up taken in the gut. This could explain why these cell populations were not increased in control animals that were never fed OVA, although future research is necessary to elucidate the functional reason for those cell populations to expand.

The intestinal immune response to OVA showed interesting differences between the animals that received OVA alone or with AT-1002. In both the small intestine and the colon the physiological response to OVA, when previously fed, is to produce IL-10 and TGF-β, both indispensable cytokines in the induction of oral tolerance. In contrast, IL-10 production did not occur in the small intestine and the colon when the antigen was fed under increased small intestinal permeability. This suggests that the decision between tolerance vs. immunity is likely taking place in the small intestine and that the immune cells activated by OVA can relocate to other mucosal sites as effector cells, where they can initiate a chronic inflammatory response. This may also explain the pathogenesis of other autoimmune diseases like diabetes type 1, where it has been shown that reducing small intestinal permeability prevented the destruction of pancreatic β-cells (35).

We believe that these data support our hypothesis in that an increase in small intestinal permeability changes the immune response to an antigen presented orally, by involving the systemic immune system (normally ignorant to dietary antigens) and by preventing the production of anti-inflammatory cytokines

throughout the intestinal mucosa. Coupled with our previous data in IL-10$^{-/-}$ mice, prevention of oral tolerance induction may be one of the mechanisms by which small intestinal permeability modulates the severity of colitis in this mouse model.

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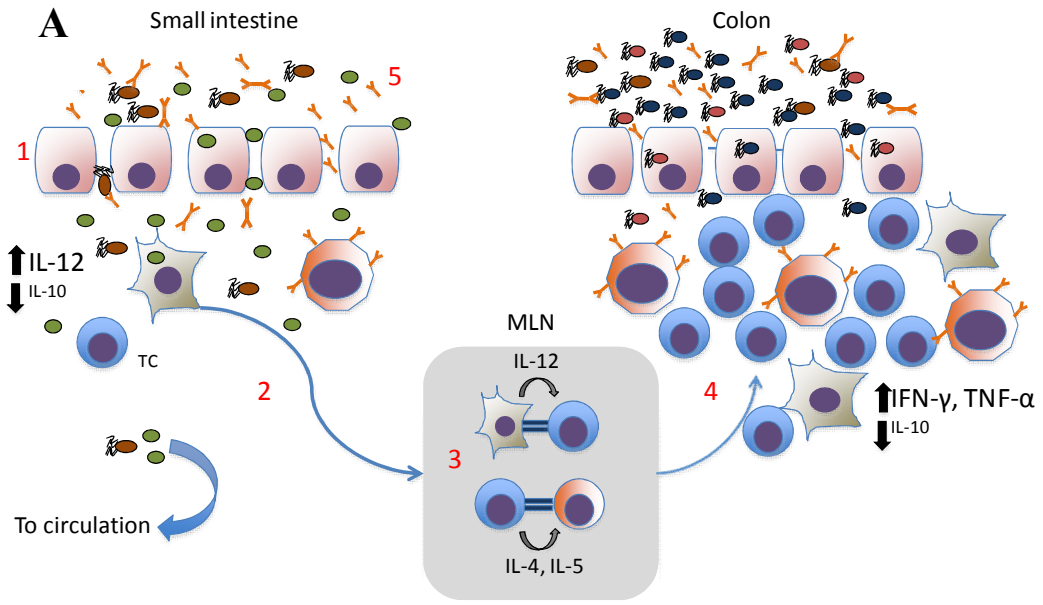
Chapter 6

General Discussion

The small intestinal mucosa acts as the first window of interaction between the external environment and the host. This large surface area has adapted to digest and absorb nutrients from an incredibly complex mixture of substances and microorganisms, some of which have the potential to cause harm to the host if given the opportunity. This difficult task is accomplished by acting as a semi permeable surface, one that efficiently absorbs water and micronutrients transcellularly and paracellularly, and excludes most other luminal components. Unlike the transcellular route, the paracellular route is not regulated by specific receptors, pumps and channels. Instead, its barrier capability is based on charge and size selectivity, and the physiological pathways that control the paracellular route are just beginning to be elucidated. By measuring paracellular permeability it has been established that it is augmentated in certain pathologies, including IBD. Although it is currently unknown exactly how this disease originates, there are several requirements that need to be present: intestinal bacterial antigens and an aberrant immune response towards them. The work presented in this doctoral thesis allows me to conclude that increased paracellular small intestinal permeability also has a key role in the pathogenesis of colitis in the IL-10$^{-/-}$ mouse and that it is not just an early manifestation of disease (Figure 6.1).


A
Small intestine
Colon
1
5
IL-12
IL-10
TC
2
MLN
IL-12
3
IL-4, IL-5
4
IFN-γ, TNF-α
IL-10
To circulation

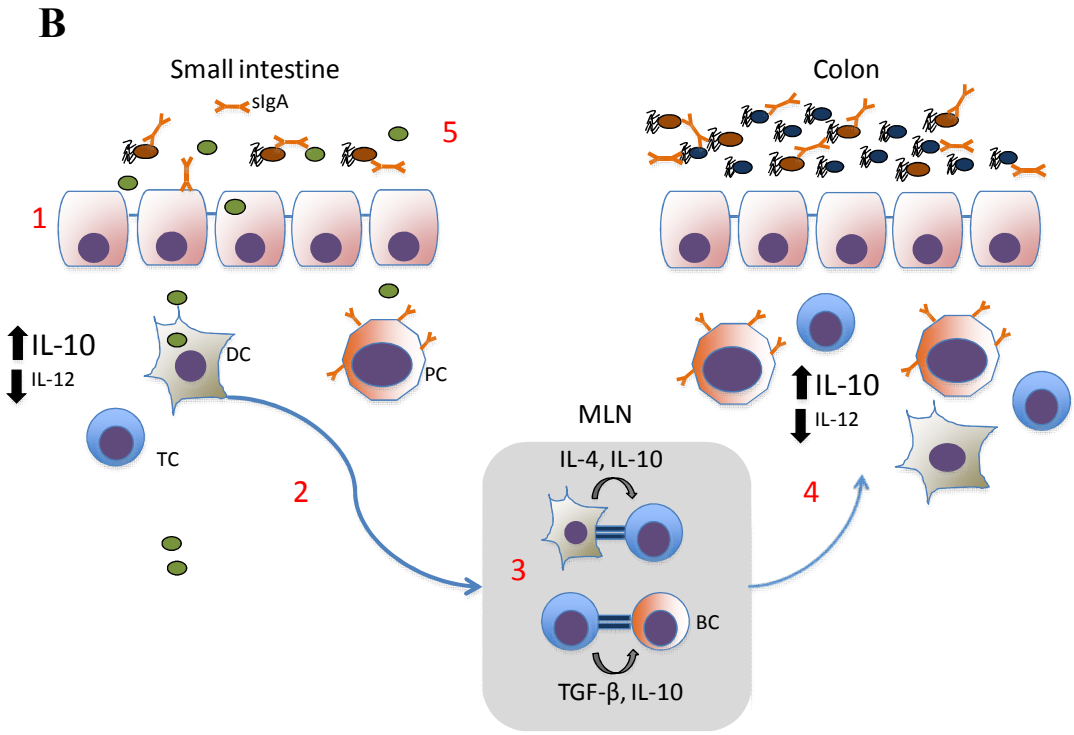

B
Small intestine
sIgA
5
1
IL-10
IL-12
DC
PC
TC
2
MLN
IL-4, IL-10
3
BC
TGF-β, IL-10
4
IL-10
IL-12
Colon

Figure 6.1 Proposed role of small intestinal permeability in the pathogenesis of colitis. A. In a healthy host with normal small intestinal permeability, antigen sampling occurs via classical routes (1) which induces the secretion of tolerogenic signals by IECs. DCs with up taken antigen travel to the MLN (2) where they encounter naïve T cells. In the MLN, antigen presentation takes place in a cytokine milieu rich in IL-10 and IL-4, committing T cells into Tregs or Th2-type T cells (TC). These T cells activate B cells (BC) into IgA- producing PCs (3). Activated T and B cells migrate back to the small intestine and to the colonic lamina propria (4), where they contribute by secreting tolerogenic signals. PCs produce large amounts of IgA, which gets assembled in its dimeric sIgA form and transported through the IEC into the lumen, helping to keep the microbial flora 'in check' (5).

B. In a genetically predisposed host unable to produce IL-10 and with increased small intestinal permeability antigen crosses the intestinal epithelial barrier with ease (1) which induces secretion of pro-inflammatory signals by IECs. DCs travel to the MLN (2) and present food and microbial antigens to TCs in the MLN in a milieu rich in IL-12, committing the TC to Th-1 type T cells. BCs are also activated by TCs or antigens themselves and become PCs (3). T cells and PCs migrate to the small intestinal and colonic mucosae. In the colon, pro-inflammatory cytokines injure the epithelial barrier, facilitating entry of microbes and initiating inflammation (4). PCs produce high amounts of monomeric IgA which is able to 'escape' paracellularly into the lumen before it becomes sIgA, hindering its function and possibly altering the colonic microbial ecology (5).

We also propose that increased permeability in the small intestinal mucosa may have an initiating role in the pathogenesis of intestinal and extraintestinal disease in humans. The exciting observation that selectively decreasing small intestinal permeability attenuates colitis in a well established mouse model supports our hypothesis and opens the possibility of using the small intestinal mucosa as a target organ to treat pharmaceutically in order to ameliorate disease in the colon. In fact, these efforts have already begun and one of the drugs used in our experiments (AT-1001) is currently being tested in pharmaceutical trials. Recent clinical studies with this drug demonstrated that it prevents some of the immune related events triggered by a gluten challenge in patients with celiac disease, suggesting its potential as a treatment for celiac disease, a disorder that does not have a pharmaceutical treatment yet (1).

Elucidating how events in the small intestinal mucosa can lead to disease in the colon, or elsewhere may also provide us with novel approaches to treat disease. We showed that increasing intestinal permeability changes the colonic flora of IL-$10^{-/-}$ and wild type mice. It is unknown whether these changes were permanent or just reflected the temporary increase in permeability induced by AT-1002. However, even temporary changes such as antibiotic or probiotic treatment can be significant in the health of the host.

Another important observation was the significant increase in total IgA secretion after AT-1002 treatment, both in IL-$10^{-/-}$ and wild type mice. The role of IgA in the gut has always been perceived as protective to the host, yet we showed in the IL-$10^{-/-}$ mouse that an increase in IgA secretion is associated with an increase in

colonic inflammation and a change in microbial flora. Although our experiments did not address whether IgA mediated those changes, it is interesting to speculate that a significant increase in IgA secretion due to abnormally increased paracellular permeability might be detrimental to the maintenance of gut homeostasis. As mentioned in a previous chapter, the leak of unassembled monomeric IgA into the lumen may reduce the amount of dimeric sIgA and its functional ability to act as a first line of defense towards luminal microorganisms.

Finally, our experiments suggest that another mechanism by which increasing small intestinal permeability modulates colonic disease is by altering the mechanisms that induce oral tolerance. As explained before, oral tolerance is the default response to the vast majority of antigens in the gut and it can be achieved by many immune mechanisms. It was very exciting to observe that just increasing small intestinal permeability, in the absence of inflammation, would change the immune response towards an innocuous antigen. This also supports our hypothesis in that the intestinal mucosa has an essential role in the decision of immunity vs. tolerance. Although increasing small intestinal permeability is not sufficient to induce a cell mediated response towards the soluble dietary antigen OVA in a healthy host, it might prove sufficient in an immune deficient host unable to properly downregulate an inflammatory response towards an otherwise innocuous antigen.

Like most scientific studies, the ones presented in this thesis are not without limitations. First, although we speculate that the increase in small intestinal permeability is due to the defective interactions between bacteria and the

epithelial barrier of IL-10$^{-/-}$ mice, this was not shown experimentally. Comparing intestinal permeabilities *in vivo* between germ-free IL-10$^{-/-}$ mice and germ-free wild type mice would show if the presence of microbes accounts for the elevation in small intestinal permeability, or if perhaps the lack of IL-10 also contributes to the increase in permeability.

Second, our oral tolerance experiments indicate that increasing small intestinal permeability changes the cytokine secretion patterns in the colon and we speculated that this is due to the migration of activated T and B cells to the colon. However, we did not show that these cells can travel to the colon. An adoptive transfer assay with radioisotope-labeled T cells isolated from the small intestine of AT-1002-treated mice into naïve mice would show if these cells have the capacity to relocate to the colon and other mucosal sites.

Third, our oral tolerance experiments were performed with a dietary antigen mainly because of the necessity to use an antigen that had never been in contact with the mice. A more realistic experiment would involve a particulate antigen of microbial origin with the ability to induce a stronger immune response. Finding an antigen with all of these characteristics is not an easy task because it is virtually impossible to know all of the microbial antigens already present in the mouse's intestinal flora. An antigen from a protozoan or a helminth may be a good option as most mice raised under SPF conditions have never been exposed to these parasites.

Another limitation of our study is the lack of taxonomic information from the microbial flora of the AT-1002 treated animals. Future research with more robust genetic fingerprinting techniques, such as pyrosequencing, should be carried out to characterize what type of bacterial flora changes are induced by increasing small intestinal permeability, and more importantly, its functional effect to the host's health.

6.2 Future Directions

Future research should address whether small intestinal permeability also has a critical role in the pathogenesis of other extraintestinal diseases, similar to what has been shown in the rat model of autoimmune diabetes (2).

Further research should also address whether decreasing small intestinal permeability can attenuate active colitis. We showed that mice treated with AT-1001 before disease was expected to occur was effective in decreasing inflammation, but in reality IBD patients never seek treatment before the onset of disease. Future experiments should test whether AT-1001 is effective in treating active colitis in mice.

As mentioned before, AT-1001 is currently being tested in clinical trials for celiac disease treatment. A similar trial should be performed to test its efficacy in IBD, both as a treatment of active disease or to extend disease remission periods in IBD patients.

6.3 Conclusions

In conclusion, the paracellular pathway between intestinal epithelial cells has become important in our understanding of gastrointestinal and systemic disease. Long thought to be a static non-regulated barrier to the passage of luminal material, it is now recognised to be a dynamic constantly changing structure with a functional state that is carefully regulated. Abnormal function of this pathway is associated with a variety of pathological states. The work from this doctoral thesis strongly suggests that in IBD, increased small intestinal permeability is involved in a causal manner in the genesis of disease and efforts to correct this permeability defect may prove efficient as a treatment of disease.

6.4 Bibliography

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