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THE ROLE OF MONOCYTE/MACROPHAGES IN IMMUNE-MEDIATED EPITHELIAL PATHOPHYSIOLOGY: IMPLICATIONS FOR INFLAMMATORY BOWEL DISEASE

BY

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A Thesis Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements for the Degree

Doctor of Philosophy in Medical Sciences

McMaster University

EPITHELIAL PATHOPHYSIOLOGY INDUCED BY MONOCYTE/MACROPHAGES

DOCTOR of PHILOSOPHY (2000)
(Medical Science)
McMaster University
Hamilton, Ontario

Title:	The Role of Monocyte/Macrophages in Immune-mediated Epithelial Pathophysiology: Implications for Inflammatory Bowel Disease
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Number of Pages: xv, 171

ABSTRACT

Crohn's disease is characterized by altered epithelial physiology including ion secretion and disrupted epithelial barrier. It has been suggested that the pathophysiology and epithelial damage in Crohn's disease may be due to inappropriate immune reactions. The purpose of my studies was to examine the role of cells of the monocyte/macrophage lineage in mediating epithelial physiology.

Confluent monolayers of the T84 colonic epithelial cell line were co-cultured with human monocyte/macrophages (peripheral blood monocytes [PBM], or lamina propria mononuclear cells [LPMC]) from controls or patients with crohn's disease ± the activating agents, bacterial lipopolysaccharide (LPS) and/or the bacterial tripeptide, f-met-leu-phe (fMLP). Monolayers were then removed and epithelial secretory (baseline short circuit current [Isc] and ΔIsc to 10^{-5} forskolin) and barrier transepithelial resistance [TER]) were measured 48h later in the Ussing chambers. Epithelial functional parameters were unchanged after co-culture with non-activated PBM. Addition of activating agent (LPS or LPS/fMLP) significantly increased Isc and reduced TER with PBM from both control and Crohn's disease. No pathophysiology occurred after co-culture with control LPMC ± LPS. However, non-activated LPMC from patients with Crohn's disease spontaneously induced changes in T84 physiological parameters similar to those produced by activated PBM. A non-pathogenic control strain of *E. coli*, added to the luminal compartment of the culture well, induced comparable changes in epithelial physiology in the presence of PBM.

The role of Tumor Necrosis Factor α (TNFα) in the regulation of epithelial secretory

and barrier functions was examined. TNFα production was significantly increased by activated PBM and non-activated LPMC from Crohn's disease. The epithelial abnormalities could be successfully abrogated by inclusion of a neutralizing antibody to TNFα.

These studies show that monocyte/macrophages are potent immune cells which may mediate some of the pathophysiology in inflammatory disorders of the gut. TNFα is a key factor mediating the monocyte/macrophage induced epithelial pathophysiology.

ACKNOLEDGEMENTS

I would like to express my gratitude to my supervisor, Dr. Mary Perdue, for her continous help, encouragement and support throughout my research. Her patience and kind comments have always provided me with the motivation to continue with the ups and downs of my research. Thanks are also extended to the members of my supervisory committee, Dr. M. Jordana, Dr. G. Sorger and Dr. D.M. Mckay for their support and excellent advice through the progression of my work.

I am also grateful to all the wonderful people in Dr. Perdue's laboratory who have been my friends and provided me with unforgetable support. I specially thank Pam for all her patience and technical advice throughout my time in the lab.

I would like to thank the very special people who have made this possible. Thanks to my parents and my sisters for all their love, support and encouragement. Finally, special thanks to my wonderful husband, Javad, and my sons, Mahdi and Mohammad. Their patience, love and continous understanding were instrumental for successful completion of my studies. It is great to share all of this with you.

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FORMAT AND ORGANIZATION OF THESIS

This thesis is organized in the "Open-faced" sandwich format, approved by McMaster University. This thesis is comprised of 5 chapters. The first chapter is a general introduction. Chapters 2 and 4, are published manuscripts in peer reviewed scientific journals. Chapter 3 is a manuscript submitted for publication. Chapter 5 is a general discussion highlighting the significant contributions of this thesis. The expanded description of selected methods are included in the appendix.

Contributions to Multi-Authored Papers

Chapter 2: Monocyte/macrophages evoke epithelial dysfunction: indirect role of tumor necrosis factor α (1998).

Authors: M Zareie, DM McKay, GG Kovarik and MH Perdue.
Journal: American Journal of Physiology, 275: C932-C939.
Comment: The experiments were conducted and data collected by the author M Zareie with the aid of GG Kovarik (completing requirements for an undergraduate thesis) under the supervision of Dr. MH Perdue.

Chapter 3: Monocyte/macrophage activation by normal bacteria and bacterial products: implications for epithelial pathophysiology in Crohn's disease (2000).

Authors: M Zareie, PK Singh, EJ Irvine, PM Sherman, DM McKay and MH Perdue.
Journal: American Journal of Pathology (submitted).
Comment: The experiments were conducted and data collected by the author M Zareie with the aid of PK Singh (technician in Dr. MH Perdue's laboratory) under the supervision of Dr. MH Perdue.

Chapter 4: Improved effects of novel glucocorticosteroids on immune-mediated epithelial pathophysiology (1999).

Authors: M Zareie, R Brattsand, PM Sherman, DM McKay and MH Perdue.
Journal: Journal of Pharmacology and Experimental Therapeutics, 289: 1245-1249.
Comment: The experiments were conducted and data collected by the author M Zareie, under the supervision of Dr. MH Perdue.

CHAPTER 1

INTRODUCTION TO THE THESIS

INTRODUCTION

1.1 INTESTINAL MUCOSA

The intestinal epithelium covers the mucosa and forms an interface between the external and internal environments in the gastrointestinal tract. Beneath the single layer of epithelial cells there is a sophisticated immune system called the gut-associated lymphoid tissue (GALT). GALT is a general term for immunoreactive cells distributed throughout the intestine which can be divided into three compartments:

1) Peyer's patches are the organized mucosal lymphoid follicles located in the wall of the small intestine. They are composed of multiple B cell follicles containing large numbers of IgA secreting B cells. The intra-follicular regions contain mature macrophages and T cells including both $CD4^+$ and $CD8^+$ subsets with generally higher numbers displaying activation markers compared to cells in the spleen or lymph nodes (Targan *et al.*, 1995; Taguchi *et al.*, 1990). In the follicle-associated epithelium of the Peyer's patches, specialized epithelial microfold (M) cells (Neutra, 1998; Neutra *et al.*, 1996; Jones *et al.*, 1994) are present which play an important role in antigen sampling, through active transepithelial transport from the lumen directly to lymphocytes to the Peyer's patches (Amerongen *et al.*, 1992).

2) The lamina propria contains a network of connective tissue located directly beneath the epithelium and a large variety of immune cells including macrophages, T cells (mainly $CD4^+$ expressing high levels of T cell receptor α/β), highly differentiated B cells (mostly IgA secreting), dendritic cells and mast cells.

Human lamina propria macrophages have been found to express a range of markers including CD68, CD33, MHC class II, and 25F9 (induced during monocyte maturation), with minimal expression of CD14 (this will be discussed further) (Nagashima *et al.,* 1996; Grimm *et al.,* 1995). Compared to peripheral T cells, lamina propria T cells are highly differentiated cells that have a reduced capacity to proliferate but a greater ability to produce cytokines (Farstad *et al.,* 1994). When lamina propria T cells are stimulated (with phorbol myristate acetate or via the CD2 pathway), they secrete high levels of cytokines consisting of a mixture of T helper 1 (IFNγ) and T helper 2 (IL-4 and IL-5) cytokines (Taylor *et al.,* 1997; Perdue and McKay, 1996; Targan *et al.,* 1995; Taguchi *et al.,* 1990; Pirzer *et al.,* 1990). In recent years, many studies have confirmed that intestinal epithelial physiology is modulated by a number of immune cells including T cells. However, considerably less is known about the ability of lamina propria cells of the macrophage lineage to directly affect epithelial function.

3) Intraepithelial lymphocytes (IEL) are located between the absorptive enterocytes, above the basal lamina. Almost all IEL are T cells and unlike those in the lamina propria, the majority of them express CD8 and are T cell receptor α/β^+,

but a proportion of them are γ/δ^+. They proliferate poorly and have cytotoxic characteristics (Yamamoto *et al.,* 1993; Mosley *et al.,* 1991). They are also capable of producing Th1 type cytokines that can affect epithelial function. IEL may be involved in the regulation of oral tolerance (Lundqvist *et al.,* 1996; Shanahan, 1994).

1.2 INTESTINAL EPITHELIUM

The intestinal epithelium is not only the site for digestion and absorption of various essential nutrients, but it also provides an effective barrier against potentially harmful substances and microorganisms in the gut lumen. The epithelium is mainly composed of a single layer of columnar enterocytes, with goblet cells and enteroendocrine cells distributed between them. A homogeneous population of stem cells in the crypt gives rise to the multiple cell types of the intestinal epithelium. The cells continuously migrate upward along the crypt-villus axis as they mature and form the absorptive enterocytes and the mucus secreting goblet cells (Kato and Owen, 1999; Goodlad, 1989). Enterocytes have numerous microvilli on their luminal surface, greatly increasing their surface area. Goblet cells are producers of mucin and are located on villi and in crypts. Interspersed between the enterocytes are enteroendocrine cells which contain a number of secretory granules and release gastrointestinal hormones (secretin, somatostatin, neurotensin, etc...) in response to changes in the luminal environment. Progenitors

from stem cells also migrate downward to the crypt base, where they form Paneth cells and some enteroendocrine cells. Paneth cells have various secretory granules that contain lysozymes, tumor necrosis factor α, and cryptidins, which are defensin-like molecules that may function to prevent proliferation of microorganisms in the crypt (Ouellette, 1997).

1.2.1 Epithelial Ion Transport

One of the main functions of the intestinal enterocytes is vectorial transport of nutrients and ions. In general, the crypt epithelium is responsible for ion secretion, and the villus epithelium is predominantly responsible for absorption. Transport across the epithelial barrier involves both transcellular and paracellular pathways. Transcellular processes are mainly active and involve the movement of molecules and ions across the apical membrane by means of specialized transporters and channels. The glycocalyx contains various enzymes and nonenzymatic proteins (receptors and transport proteins) necessary for digestion and absorption of nutrients (Frey *et al.,* 1996; Maury *et al.,* 1995). Following digestion of proteins and carbohydrates, amino acids and monosaccharides are transported across the apical membrane barrier by sodium-linked co-transporters, and some peptides are absorbed by proton-linked transporters (Alpers, 1994; Heyman *et al.,* 1990). Enterocytes can also take up larger intact proteins by endocytosis. Most proteins absorbed by this route undergo digestion within

lysosymes. It has been shown that a small quantity of intact proteins bypass lysosomal degradation by binding to specific receptors on the apical membrane of the enterocytes (Heyman *et al.,* 1982). This might be of particular importance in immune or inflammatory responses of the epithelium.

Secretion of ions and water is an important physiological process. It provides water to solublize nutrients, and wash away harmful substances and microbes. It also provides ions necessary for the function of various co-transporters and nutrient absorption. There is normally an inward gradient for Na^+ and an outward gradient for K^+ through the function of Na^+/K^+ ATPase pump located at the basolateral membrane of the enterocytes. In villus epithelium, water crosses the tight junctions passively by solvent drag according to the osmotic gradient created by Na^+ absorption. In the crypt cells, a higher intracellular concentration of Cl^- is created by the $Na^+/K^+/Cl^-$ co-transporters located in the basolateral membrane. Upon stimulation, Cl^- channels in the apical membrane open and an outward flow of Cl^- ions followed by passive flow of Na^+ ions and water through the tight junctions is created, resulting in a net secretion of ions and water (Kaunitz *et al.,* 1994; Chang and Rao, 1994).

1.2.2 Epithelial Barrier Function

The intestinal epithelium provides an effective barrier against antigens, harmful macromolecules and microorganisms in the gut lumen. The ability of the

epithelium to exclude noxious substances is enhanced by physical defense mechanisms associated with mucosal surfaces and the structure of the enterocyte itself. The adjacent enterocytes are connected at their apices by junctional complexes mainly, including tight junctions (zonula occludens), adherence junctions (zonula adherens), and desmosomes (macula adherens) (Madara and Trier, 1994). The function of the tight junctions is an important component of the intestinal barrier. The tight junctions form a continuous seal with highly regulated gates that open and close in response to signals from the surrounding environment, allowing passage of ions and water but excluding peptides and macromolecules (as small as 1900 Da) with antigenic potential (Madara and Trier, 1994). Goblet cells secrete mucin that shields the mucosal epithelial cells from direct contact with the luminal environment (Cone, 1999). Paneth cells produce defensins that target microorganisms (Ouellette and Selsted, 1996). The glycocalyx of the epithelium serves as a diffusion barrier that prevents direct contact of most particles, bacteria, viruses and harmful macromolecules with the microvilli (Frey *et al.*, 1996). The epithelial barrier is dynamic by nature, and tightly regulated by a number of physiological mediators including cytokines, lipids and metabolites (Perdue and McKay, 1994).

1.3 CROHN'S DISEASE

Crohn's disease is a chronic inflammatory bowel disease (IBD) of unknown

etiology. The mean annual incidence is highest among those aged 15 to 30 years, with 25-35% of the patients developing the disease before age 20. Most studies through the world have reported a rise in the incidence of the disease over the last 50 years particularly in the western world. Increased incidences have also been reported for some ethnic groups including Jews (Binder, 1995).

Crohn's disease is characterized by a chronic transmural granulomatous inflammation of the gastrointestinal tract. The inflammation can affect the entire gastrointestinal canal, from mouth to anus. The most commonly affected area is the ileocaecal region, followed by colon and small bowel. The clinical signs and symptoms of patients with Crohn's disease include recurrent bowel obstruction, abscesses, fistulae and sepsis (Duchmann and Zeitz, 1999). In addition, Crohn's disease is also associated with many extra-intestinal manifestations including arthritis and arthralgia (Grant and Husby, 1992), perianal lesions, liver complications and skin and eye lesions (erythema nodosum, uveitis) (Greenstein *et al.,* 1976), which may be present in one third of the patients.

The chronic nature of Crohn's disease, its early onset in life and the lack of a definite cure, makes it a disease with a relatively high morbidity in adulthood (during exacerbations of the disease). Most patients, however, will be able to adapt successfully. With regard to mortality, most studies indicate that the mortality rate for Crohn's disease is only slightly increased compared to the general population (Persson *et al.,* 1996; Ekbom *et al.,* 1992), perhaps due to an increased risk of

colon cancer in patients with colonic involvement (Ekbom *et al.,* 1990).

1.3.1 Treatment of Crohn's Disease

For a long period of time, resection of the diseased segment of the bowel has been the treatment of choice for patients suffering from Crohn's disease. Today, however, surgery is used only when medical treatment fails. Development of anti-inflammatory drugs and the increasing knowledge of the inflammatory processes involved in the course of the disease has led to the current, more conservative treatment approaches. Medical therapy is aimed generally to control the inflammatory process and is administered for active and chronically active disease as well as remission maintenance. Anti-inflammatory therapy of Crohn's disease is based on the use of glucocorticosteroids, 5-aminosalicylates and azathioprine or 6-mercaptopurine or a combination of those (Duchmann and Zeitz, 1999). Among these, corticosteroids remain the most important drugs for the treatment of active Crohn's disease (Greenberg *et al.,* 1994; Brattsand, 1990). However, the potentially serious side effects associated with corticosteroid treatment, such as adrenal insufficiency, hypertension and growth retardation in children (Compston *et al.,* 1987; Singleton *et al.,* 1979), has prompted the search for new topical corticosteroids that combine the advantage of high anti-inflammatory activity and a low adverse effect profile. In addition, a variety of new non-steroid agents have recently become available which do not interfere with the inflammatory

process at multiple levels like steroids do, but specifically target defined molecules. The most promising of these approaches is the use of an antibody to TNFα, a proinflammatory cytokine which has been shown to be involved in the pathophysiology of Crohn's disease (VanDeventer, 1997).

Anti-TNFα therapy

Chronic inflammatory activity in Crohn's disease may be sustained by local release of pro-inflammatory cytokines, particularly TNFα, from intestinal macrophages and other immune cells (VanDeventer, 1997). Thus, the specific blockade of TNFα has been considered a promising approach for the treatment of intestinal inflammation in Crohn's disease. Several clinical trials have demonstrated that a one time application of a monoclonal antibody against TNFα, induces complete endoscopic and clinical remission in about 40% of patients with Crohn's disease (Rutgeerts *et al.,* 1999; Targan *et al.,* 1997). These findings may indicate a pivotal role for TNFα in the pathophysiology of intestinal inflammation in Crohn's disease, which will be discussed elsewhere.

Novel Corticosteroids

The specific actions of glucocorticosteroid are mediated through triggering of glococorticosteroid receptors. These receptors seem to be uniform in the human body (Gustafsson *et al.,* 1990). This means that the same receptor type (depending

on its cellular location) can mediate therapeutic as well as adverse corticosteroid reactions. Currently, there is no possibility of separating the corticosteroid actions via subtypes of the steroid receptors. However, a way to achieve separation is to deposit a suitable quantity of the corticosteroid directly in the organ, such as corticosteroid inhalation for the topical treatment of asthma (Brattsand, 1990). Therefore, the optimal corticosteroid for topical anti-inflammatory/ immuno-suppressive therapy of IBD would be a compound with high topical corticosteroid potency and high metabolic stability to ensure the full activity in the bowel. In addition, the compound should have a high rate of first pass metabolic degradation in the liver before being distributed in the systemic circulation. Budesonide is the prototype for this new generation of glucocorticosteroids. Budesonide is characterized by its high water solubility (facilitates its dissolution and transport into the bowel wall) and a high rate (~90%) of oxidative bio-transformation on first pass through the liver, thus low bio-availability (10-15%) after rectal or oral administration (Jwell and Rutgeerts, 1993; Brattsand, 1990). More recently, newer analogs of budesonide have been developed. Compared to budesonide, these compounds display an even higher degree of steroid receptor affinity (provides higher topical anti-inflammatory activity) and a more rapid metabolization during first-pass through the liver (ensures lower systemic bio-availability and minimal systemic side effects) (Thalen *et al.*, 1998).

1.4 IMMUNE-MEDIATED EPITHELIAL PATHOPHYSIOLOGY

Inflammatory bowel diseases are often characterized by altered epithelial physiology, typically increased electrolyte secretion and permeability that can create a luminally directed driving force for water movement resulting in diarrhea. Epithelial pathophysiology may be caused by activated immune cells, since many studies have provided evidence that the transport and barrier functions of the epithelial lining of the mucosal surfaces are regulated by immune cells such as T lymphocytes, mast cells and neutrophils (Perdue and McKay, 1996) and all of these immune cells have the potential to contribute to various aspects of the disease process in IBD. Using human fetal intestinal explants to examine the effects of T cell activation (by pokeweed mitogen or antibodies against the CD3 component) on gut structure, a reduction in the surface area: volume ratio, goblet cell depletion and increased crypt cell proliferation has been observed (MacDonald and Spencer, 1988). Co-culture studies performed using epithelial monolayers and anti-CD3 activated peripheral blood mononuclear cells, have also shown the importance of T cells and their cellular interactions in the regulation of epithelial cell functions (McKay *et al.,* 1996). Mast cells are key components of the immediate hypersensitivity reactions and reactions to other antigens; the ability of activated mast cells to alter epithelial physiology has been also described (Perdue and McKay, 1994). Furthermore, experiments examining the transepithelial migration of neutrophils and its consequences on intestinal epithelial physiology, have

clearly described the potential of these cells in regulating the epithelial function (Madara *et al.,* 1993).

Although many studies such as those outlined above, have confirmed the concept of immuno-modulation of epithelial physiology, few studies have considered the ability of cells of the monocyte/macrophage lineage to directly affect intestinal epithelial physiology.

1.5 LAMINA PROPRIA MACROPHAGES

The lamina propria of the intestinal mucosa contains one of the largest compartments of cells of the monocyte/macrophage lineage in the body (Rogler *et al.,* 1998; Pavli and Doe, 1992). Macrophages form 10-20% of the mononuclear cells in the intestinal lamina propria; they are mostly accumulated beneath the epithelium, where they are likely to be exposed to a diverse range of bacteria and bacterial products present in the lumen (Chadwick and Anderson, 1990). These cells represent an important first line of defense against external environmental challenges and are believed to play an important role in mucosal immunology, including phagocytosis of exogenous pathogens, antigen presentation, and release of a number of proinflammatory and immunoregulatory mediators (Lugering *et al.,* 1998; Nathan, 1987).

1.5.1 Phenotypic Characteristics of Macrophages in Normal Mucosa

Intestinal macrophages are different from blood monocytes and macrophages found in other tissues. Normal intestinal macrophages generally lack the classical monocyte-specific surface markers CD14 and CD16 (FcγIII receptor) (Grimm *et al.,* 1995a; Mahida *et al.,* 1989a). CD14 is the receptor for lipopolysaccharide (LPS), a major product of Gram-negative bacteria which are normally present in large numbers in the lumen of the gut. CD14 is mainly expressed on circulating monocytes and alveolar macrophages, where it increases the sensitivity of these cells to LPS. Macrophages in normal intestine, however, do not express CD14 and appear to be insensitive to LPS stimulation (Grimm *et al.,* 1995b; Duchmann *et al.,* 1995b). Furthermore, less than 5% of macrophages in normal mucosa express the typical macrophage marker CD11b (complement receptor 3, CR3), and intracellular adhesion molecule (ICAM-1) is expressed by only 7% of them (Malizia *et al.,* 1991). Recent reports also suggest that there is a low expression of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) on normal colonic macrophages (Rogler *et al.,* 1998). The co-stimulatory molecules play an important role in the regulation of the type of the immune response triggered by antigen presenting cells. A lack of these molecules could lead to antigen desensitization (Thompson, 1995). This coupled with the lack of CD14 expression on intestinal macrophages could be of particular importance in the mechanisms involved in the induction of tolerance towards the luminal antigens to

which the mucosa is constantly exposed.

1.5.2 Phenotypic Changes of Macrophages in IBD

During active IBD, large numbers of monocytes leave the bloodstream and migrate into the mucosa. In active IBD lesions, these recently recruited lamina propria monocyte-like macrophages express the CD14, CD68 and L1 molecules (typical blood monocyte markers) and represent the majority of those with an increased ability to undergo respiratory burst (Rugtveit *et al.,* 1995; Baldassano *et al.,* 1993). This sub-population of macrophages that are rarely present in the normal tissue, also express surface markers indicative of an increased activation state which is associated with increased functional ability, enhanced antigen presenting activity and increased production of proinflammatory mediators (Reinecker *et al.,* 1993). In addition, macrophages isolated from active Crohn's lesions exhibit elevated expression of the co-stimulatory molecules B7-1 and B7-2, which might allow better cooperation with T cells and facilitate Th1 hyper-reactivity in Crohn's disease (Rogler *et al.,* 1998). Macrophages are also evident in early IBD lesions, as demonstrated by presence of ICAM-1$^+$ and HLA-DR$^+$ cells in aphtoid ulcers of Crohn's disease (Morise *et al.,* 1994). Therefore, cells of the monocyte/macrophage lineage appear to be involved in all stages of IBD, emphasizing their importance in pathophysiology of IBD, particularly Crohn's disease.

1.5.3 Cytokines Produced by Macrophages

An elevation of several pro-inflammatory cytokines has been demonstrated in IBD (Monteleone *et al.,* 1997; Reimund *et al.,* 1996; Kusugami *et al.,* 1995; Youngman *et al.,* 1993; Reinecker *et al.,* 1993; Stevens *et al.,* 1992; Cappello *et al.,* 1992; Mahida *et al.,* 1991). As important mediators of inflammation and regulators of inflammatory process, cytokines produced by macrophages, such as TNFα, have been implicated in the pathogenesis of IBD (Reimund *et al.,* 1996; Reinecker *et al.,* 1993; Stevens *et al.,* 1992; Cappello *et al.,* 1992).

Tumor Necrosis Factor-α

TNFα is a member of a large family of proteins and receptors that can transmit signals between immune cells and other cells. It is involved in inflammation, apoptotic cell death, metabolism, thrombosis and fibrinolysis. The secreted form of TNFα is a 17 kD, non-glycosylated protein that is mainly produced by monocytes and macrophages, upon stimulation by a variety of stimuli. Recently, an important role for TNFα as a pro-inflammatory mediator in Crohn's disease has been recognized, which has resulted in the development of therapeutic strategies that target TNFα.

Increased production of TNFα by lamina propria mononuclear cells, and organ cultures of biopsy specimens from inflamed or morphologically normal intestinal mucosa of patients with Crohn's disease has been reported. Braegger et

al. found elevated TNFα levels in the stool of children with active Crohn's disease, which returned to normal during remission (Braegger *et al.,* 1992). It was also reported that the frequency of the TNFα producing cells in the mucosa of inflamed intestine was increased, and higher levels were observed in Crohn's disease rather than ulcerative colitis (Breese *et al.,* 1994). Other studies have also reported up-regulation of TNFα production in morphologically normal intestinal biopsies from patients with Crohn's disease (Reimund *et al.,* 1996), and in adherence separated macrophages of IBD mucosa following stimulation by pokeweed antigen (Rugtveit *et al.,* 1997). In addition, the use of TNFα neutralizing antibody (a high affinity human/mouse chimeric antibody, cA2) has proven to be an effective therapeutic approach in patients with Crohn's disease (Targan *et al.,* 1997; Van Dullement *et al.,* 1995). In the most recent study by Rutgeerts et al., it was demonstrated that cA2 (Infliximab) remarkably improves the signs and symptoms of Crohn's disease in patients with moderate to severe, corticosteroid-resistant disease activity, and that the treatment was well tolerated (Rutgeerts *et al.,* 1999). These results are comparable with the very potent anti-inflammatory effects of anti-TNFα therapy in severe rheumatoid arthritis, a disease with several immuno-pathological features similar to Crohn's disease (Feldmann *et al.,* 1995).

1.6 NORMAL INTESTINAL FLORA AND IBD

It has been long recognized that normal intestinal flora is a crucial element in normal development of the gut and its function. However, the possibility that components of the normal flora could contribute to initiation or exacerbation of the pathophysiology of IBD, has intrigued investigators for years. Overwhelming data from a variety of animal model systems and clinical investigations support a pivotal role for luminal bacteria as a potent stimulus, driving the chronic intestinal inflammation in Crohn's disease. Enteric bacteria or their products have been detected within the inflamed mucosa of patients with Crohn's disease (Klasen *et al.*, 1994). Clinical experience has shown that antibiotic treatment (Peppercorn, 1993; Sutherland *et al.*, 1991; Jakobovits and Schuster, 1984) or diversion of fecal stream (Rutgeerts *et al.*, 1991) reduces the disease activity in patients suffering from Crohn's disease. The hypothesis that IBD is due to a dysregulated and exaggerated immune response to bacteria and their products normally present in the intestinal flora, has been substantially strengthened by the observations of Duchmann et al. They have shown that, unlike normal mucosa, mucosal mononuclear cells isolated from patients with IBD proliferate when exposed to autologous intestinal bacteria (Duchmann *et al.*, 1995). These results suggest that, in healthy individuals, there is tolerance towards autologous intestinal flora, and that this tolerance is lost during inflammation associated with IBD. The same investigators also demonstrated that animals are tolerant to their own flora in health

but not after development of experimental colitis (Duchmann *et al.*, 1996). Finally, the spontaneous colitis that occurs in some murine transgenic and knockout models of colitis does not occur when the animals are kept in germfree conditions (Sellon *et al.*, 1998; Contractor *et al.*, 1998; Dianda *et al.*, 1997; Rath *et al.*, 1996). Despite the substantial body of evidence on the association of commensal flora and gut inflammation in Crohn's disease, no information is currently available on the role of commensal bacteria in altering epithelial function.

1.7 OBJECTIVES OF THESIS RESEARCH PROJECT

Crohn's disease is generally characterized by altered epithelial physiology including increased ion secretion and disrupted epithelial barrier. Many studies have confirmed the concept of immuno-modulation of epithelial function (Perdue and McKay, 1996), and it has been suggested that the pathophysiology and epithelial damage in Crohn's disease may be due to inappropriate or exaggerated immune reactions (Duchmann *et al.,* 1996; Schreiber *et al.,* 1995; MacDonald, 1995; Kuhn *et al.,* 1993). Very few studies, however, have considered the ability of cells of the monocyte/macrophage lineage to directly affect epithelial function. The specific aims of this research project were:

2) To develop an *in vitro* co-culture model to investigate the role of monocyte/macrophages in mediating epithelial physiology.

3) To identify the key factors(s) involved in the monocyte/macrophage-induced epithelial functional abnormalities.

4) To compare the cells of the monocyte/macrophage lineage from healthy volunteers *versus* those obtained from patients with Crohn's disease in their ability to alter epithelial physiology upon activation by bacteria/bacterial products.

5) To examine if non-pathogenic control bacteria can induce epithelial pathophysiology *via* monocyte/macrophage activation.

6) To investigate the efficacy of two recently developed glucocorticosteroids,

D5519 and S1316, to inhibit the monocyte/macrophage-mediated epithelial physiological changes.

CHAPTER 2

MONOCYTE/MACROPHAGES EVOKE EPITHELIAL DYSFUNCTION: INDIRECT ROLE OF TUMOR NECROSIS FACTOR α

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American Journal of Physiology, 275 (Cell Physiology 44): C932-C939, 1998.

2.1 ABSTRACT

We examined the ability of peripheral blood monocytes (PBM) activated by bacterial products to alter epithelial physiology. Confluent monolayers of the T84 colonic epithelial cell line were grown on filter supports and then co-cultured in the presence of human PBM ± the activating agents, bacterial lipopolysaccharide (LPS) and the bacterial tripeptide, f-met-leu-phe (fMLP). After 24 or 48h, monolayers were mounted in Ussing chambers where parameters of epithelial function were measured. Exposure to activated PBM resulted in a significant increase ($p<0.05$) in baseline short-circuit current (250% after 48h) that was associated with enhanced secretion of Cl^- ions. In addition, epithelial permeability was significantly increased as shown by reduced transepithelial resistance and increased flux of ^{51}Cr-EDTA. Activated PBM produced substantial amounts (~3 ng/ml at 48h) of tumor necrosis factor α (TNFα). TNFα was identified as a key mediator acting via an autocrine mechanism to induce epithelial pathophysiology. Our data show that PBM, when activated by common bacterial components, are potent effector cells capable of initiating significant changes in the transport and barrier properties of a model epithelium.

2.2 INTRODUCTION

Mucosal surfaces, particularly the intestine, are exposed to a wide variety of commensal and potentially pathogenic bacteria. Several lines of evidence now point to a role for bacteria and/or their products in the pathogenesis of mucosal inflammatory disorders (11,41), especially in the context of aberrant immune function (10,18). Inflammatory bowel diseases (IBD) are often characterized by altered epithelial physiology, typically increased permeability and electrolyte secretion that can create a luminally-directed driving force for water movement resulting in diarrhea. Epithelial pathophysiology may be caused by activated immune cells, since many studies have provided unequivocal evidence that the transport and barrier functions of the epithelial lining of mucosal surfaces are regulated by cells such as lymphocytes, mast cells and neutrophils (29,30).

Although many studies have confirmed the concept of immuno-modulation of epithelial physiology, few studies have considered the ability of cells of the monocyte/macrophage (PBM) lineage to directly affect epithelial function. However, these cells are among the first immune cells to react on initial exposure to antigens and infective organisms. For instance, monocytes exposed to lipopolysaccharide (LPS) and the bacterial tripeptide, formyl-Met-Leu-Phe (fMLP), respond with the production of mediators and the synthesis of cytokines/growth factors such as the interleukins (IL), IL-1, IL-6, tumor necrosis factor α (TNFα) and transforming growth factor β (TGF β) (1,8,16,27,44). These factors can directly, or indirectly,

affect epithelial function, altering transport and barrier characteristics (24,25,38,49). In addition, processes from tissue macrophages occur close to the basement membrane of the overlying epithelium and this spatial association may facilitate bi-directional communication between the two cell types. Finally, monocyte-chemoattractant peptide-1 (MCP-1) has been immunocytochemically demonstrated in the surface epithelium of human colonic biopsies and its expression is enhanced in tissues from patients with Crohn's disease or ulcerative colitis (35). These findings indicate a clear potential for PBM to regulate epithelial physiology.

Resident intestinal macrophages do not normally express the lipopolysaccharide (LPS) receptor, CD14 (22). However, it was recently reported that macrophages in resected intestinal segments from patients with IBD express a unique phenotype with unusually high levels of CD14 (12,13), presumably due to rapid recruitment of monocytes from the circulation to the gut (4,39). Similarly, it has been shown that monocytes are recruited to the airways during an inflammatory response and these newly recruited cells are more active in tissue damage than resident macrophages (6).

Integrating these themes, this study examined the specific hypothesis that human PBM activated by the bacterial products, LPS and fMLP, can influence epithelial electrolyte transport and barrier functions. Here, we utilized the human colonic T84 cell line as a model epithelium (9) and a co-culture approach analogous to that used to define the ability of lymphocytes and polymorphonuclear cells

(PMNs) to regulate epithelial physiology (19,24). Our data show that purified PBM (in the absence of other classes of immune cells) significantly increased luminally-directed Cl^- secretion and disrupted epithelial barrier function. These changes in epithelial function were inhibited by inclusion in the co-culture system of a neutralizing antibody against TNFα, implicating this cytokine as a critical mediator in gut pathophysiology. Further studies indicated an important autocrine mechanism of action for TNFα on PBM. Thus, PBM have been identified as being capable of directly modulating epithelial function. We speculate that given appropriate environmental conditions, activation of PBM could be a precipitating event in the onset of pathophysiology leading to chronic secretory or inflammatory disease in the intestine.

2.3 MATERIALS AND METHODS

2.3.1 Cell Culture

Epithelial Cells

T84 cells (passage 45-65) were seeded onto tissue culture-treated semi-permeable filter supports (0.4 μm pore size, 1.0 cm^2 surface area; Costar Corporation, Cambridge, MA) at a concentration of 10^6 cells/mL and grown in culture media consisting of equal volumes of Dulbecco's modified eagle medium (DMEM) and F12 medium, supplemented with 1.5% (v/v) HEPES, 2% (v/v) penicillin-streptomycin and 10% newborn calf serum (all from Gibco Laboratories, Grand Island, NY) (24). After culture for 7 days, confluent T84 monolayers consistently displayed electrical resistances $\geq$1,000 $\Omega.cm^2$.

Immune Cells

Human peripheral blood mononuclear cells (PBM) from healthy volunteers (male and female, ages 23-45) were isolated by one-step density centrifugation of whole blood over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and resuspended in fresh media at 10^6 cells/mL. The PBM population was obtained by plastic plating of PBM (4 h at 37°C) and subsequent removal of non-adherent T and B cells. Fresh media was added to the adherent cells, which were then incubated for 18 h at 37°C prior to use in co-culture studies. Assessment of T cells and

monocytes in the adherent cell population was carried out by two color flow cytometry analysis (FACS) after staining the cells with FITC-conjugated anti-CD3 (OKT3, Coulter Immunology Corp., Hialeah, FL) and PE-conjugated anti-CD14 (Caltag Laboratories, San Francisco, CA), respectively. Analysis was performed using FACScan (Becton Dickinson, Mississauga, ON, Canada) followed by data analysis using PC-Lysys II computer software (Becton Dickinson).

2.3.2 Immune Cell Activation

PBM were activated by addition of *Salmonella minesota* lipopolysaccharide (LPS, 10 µg/mL) and *Escherichia coli*-derived tripeptide (formyl-Met-Leu-Phe, fMLP, 0.1 µM) (both from Sigma Chemical Co., St. Louis, MO) to the culture media prior to co-culture. Activation was indicated by production of TNFα measured in conditioned medium (CM) by an ELISA assay (Biotrack, Oakville, Ontario, Canada). The sensitivity of the assay was 4 pg/ml. IL-2 (a marker of T cell contamination) was assessed in the same samples by ELISA (Advanced Magnetics, Cambridge, MA).

2.3.3 Co-Culture Studies

Confluent T84 monolayers were co-cultured for 24 or 48h with (LPS/fMLP)-activated PBM (25,000 - 200,000 cells/well; unless stated otherwise $2x10^5$ PBM were used) placed in the basal compartment of the co-culture wells. Control groups

included 1) T84 monolayers, 2) T84 monolayers cultured with LPS/fMLP, and 3) T84 monolayers cultured with non-activated PBM. Some experiments were conducted with naive T84 monolayers exposed to 50% CM for 48h (prepared by culture of PBM with LPS/fMLP for 24h).

2.3.4 Ussing Chamber Studies

Epithelial Ion Transport

Following co-culture, T84 monolayers were mounted in Ussing chambers as previously described (24). Epithelial monolayers were bathed in oxygenated Krebs buffer (37°C), containing 10 mM glucose as an energy source in the serosal buffer which was osmotically balanced by 10 mM mannitol in the mucosal buffer. The epithelial spontaneous potential difference (PD) was maintained at zero volts by the continuous injection of an external current by an automated voltage clamp (World Precision Instruments Inc, Sarasota, FL). This short-circuit current (Isc, in $\mu A/cm^2$) reflects net active ion transport across the preparation. Baseline Isc was recorded after a 15 min equilibration period. Stimulated ion secretion was measured by addition of the cholinergic agonist, carbachol (10^{-4} M), or the adenylate cyclase-activating agent, forskolin (10^{-5} M), (both from Sigma Chemical Co.), to the serosal side of the T84 monolayers and recording the maximum increase in Isc (24).

The mucosal-to-serosal (M→S), serosal-to-mucosal (S→M), and net fluxes of ^{22}Na and ^{36}Cl were determined using standard methodologies. Briefly, after T84

monolayers had established a stable baseline Isc, ^{22}Na and ^{36}Cl were added to either the serosal or mucosal buffer at final concentrations of 4 μCi/mL for ^{22}Na and 2 μCi/mL for ^{36}Cl. Following a 20 min equilibration period, samples (50 μl) were taken from the "hot" buffers for calculation of the tracer specific activity. One mL samples were obtained from the cold buffer at 15 minutes intervals and replaced with appropriate Krebs buffer. Radioactivity in each sample was measured in a γ-counter and a scintillation counter and flux rates were calculated using standard formulae (42).

Epithelial Permeability

Electrical resistance is a measure of the barrier property of the epithelium to passive ion movement. At intervals during each experiment, PD across the monolayer was clamped at 1.0 mV (differential pulse method, 1 pulse/30 seconds), and the resulting change in current used to calculate the transepithelial ion resistance (R, in $\Omega.cm^2$) according to Ohm's law (33). As an indication of epithelial permeability to larger molecules, the M→S movement of the inert probe, ^{51}Cr-EDTA (362.3 Da, diameter 1.15 nm) was measured. ^{51}Cr-EDTA (Radiopharmacy, McMaster-Chedoke Hospital, Hamilton, ON, Canada) was added to the mucosal buffer at a final concentration of 6.5 μCi/mL. Non-radioactive Cr-EDTA was added to the serosal buffer to maintain the osmotic balance. Fluxes were determined using 30 min flux periods (14).

2.3.5 Cell Viability

T84 monolayer viability was assessed by measuring release of lactate dehydrogenase (LDH) (20). After co-culture, T84 monolayers were removed and rinsed three times in fresh phosphate-buffered saline (PBS). Epithelial monolayers were lysed by immersing each filter in 0.1% (v/v) Triton-X 100 (Sigma chemical Co.)/PBS for 30 min at room temperature followed by vigorous manual pipetting. The lysate was centrifuged at 500 rpm for 5 min and the supernatant was analyzed for LDH activity using an automated multiple point rate test (Kodak, Rochester, NY).

2.3.6 Studies to Determine the Role of TNFα

The effect of 48h exposure to human recombinant TNFα (3 or 6 ng/ml; Centocor) on T84 function was assessed. These concentrations were chosen based on the detected levels of TNFα in CM at 24 or 48h. Additional T84 monolayers were co-cultured with PBM activated with human recombinant TNFα (≥6 ng/ml; Centocor).

The role of TNFα in the PBM-modulation of epithelial transport and barrier functions was assessed by inclusion of a neutralizing antibody to TNFα, cA2 (1μg/ml, >100 fold excess of the TNFα measured in the CM), (Centocor Inc., Malvern, PA). An irrelevant isotype matched antibody (anti-hepatoma IgG1, AF20; Centocor) was used as control. Additional studies examined the effect of cA2 in CM

added to T84 cells or to PBM.

2.3.7 Statistics

Results are presented as mean ± SEM. Due to variability in absolute values between different batches of T84 cells, data were normalized to control values in each experiment (expressed as percentage of control); n values represent the number of experiments (different blood donors) in which 2-4 monolayers were examined for each condition. Data were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls comparison. Student's t test was used where appropriate for individual comparisons. Statistically significant differences were accepted at $p < 0.05$.

2.4 RESULTS

2.4.1 Immune Cells

FACS analysis showed that >95% of the adherent immune cell population expressed CD14 and were the appropriate size for monocytes (n=6). Less than 5% of cells expressed CD3, indicating that virtually no T cells were present. PBM stimulated by LPS/fMLP secreted substantial amounts of TNFα after 24h compared to non-activated PBM (5.58±1.52 ng/mL vs 1.15±0.51 ng/mL; n=3) and 48h (3.09±1.26 vs 0.71±0.31 ng/mL; n=8). IL-2 was not detected (<4 pg/mL). Viability was >96% as measured by trypan blue exclusion following recovery of cells after plastic adherence.

2.4.2 Epithelial Physiology after Co-culture with Monocytes

T84 monolayers cultured with LPS/fMLP in the absence of PBM displayed transport characteristics that were not significantly different from control T84 monolayers cultured in media alone (data not shown). Therefore, T84 cells with no additions were used as controls in subsequent experiments.

Epithelial Ion Transport

Baseline Isc of T84 monolayers was unaltered after 24-48h of co-culture with non-activated PBM (Fig. 2.1A). In contrast, LPS/fMLP activated PBM evoked a

significant increase ($p<0.05$) in baseline Isc after 24 and 48h of co-culture to 174±14% and 251±16% of control values, respectively (Fig. 2.1A). As few as 5×10^4 cells/well caused a significant elevation ($p<0.05$) in baseline Isc following 48h of co-culture (146±21% of control values) (Fig. 2.1B).

Absolute values from one representative experiment were 0.8 ± 0.1 and 3.8 ± 0.3 $\mu A/cm^2$ for control and T84 monolayers co-cultured with activated PBM for 48h, respectively (n=4 replicates). Because Isc values are relatively insensitive in the low range (<10 μA), we measured bi-directional transepithelial fluxes of Na^+ and Cl^-. After 48h of co-culture, there was a significant increase in apically-directed flux of Cl^- across T84 monolayers compared with control values as well as an increased net flux of Cl^- (Table 2.1). Under these experimental conditions, there was no significant difference in the transepithelial movement of Na^+.

The Isc increase evoked by forskolin (88.0±9.3 $\mu A/cm^2$) was unaltered by co-culture with non-activated PBM (24-48h) or by exposure to activated PBM for 24h. However, after 48h of culture with activated PBM, there was a significantly diminished response (Fig. 2.2). In contrast, the stimulated Isc evoked by carbachol was unaffected by co-culture (24 or 48h) with PBM or activated PBM (119±24% and 95±21% of control values, respectively).

Table 2.1: Transepithelial fluxes of Na^+ and Cl^-.

	Ion Fluxes (μEq/h/cm^2)						Isc (μEq /h/cm^2)
	Na^+			Cl^-			
	M-S	S-M	Net	M-S	S-M	Net	range
Control	0.42±0.06	0.44±0.04	-0.02±0.06	0.51±0.06	0.37±0.05	0.14±0.10	0-0.2
A-PBM	0.56±0.08	0.55±0.08	0.01±0.01	0.45±0.05	0.97±0.11*	-0.52±0.05*	0.1-0.6

Control T84 monolayers or T84 monolayers with LPS/fMLP-activated monocytes (A-PBM) were cultured for 48h. Ion fluxes were subsequently measured across T84 monolayers mounted in Ussing chambers; values are mean ± SEM; n=7; * $p<0.05$ compared to control.

Epithelial Permeability

Co-culture with non-activated PBM resulted in a small drop in transepithelial resistance (from 1865±130 to 1492±112 Ω/cm^2) This change in the barrier function of the epithelium was considerably enhanced by activation of PBM. Thus, after 24h of co-culture with activated PBM, T84 resistance was reduced to 50±4% of that of time-matched controls. T84 resistance was consistently <40% of control values

after 48h of co-culture with activated PBM (Fig. 2.3A). The reduction in resistance was dependent on the number of PBM present in the culture (Fig. 2.3B), with as few as 2.5×10^4 cells/well causing a significant decrease ($p<0.05$) to 60±16% of control values after 48h of co-culture. The decrease in resistance was maximal at 2×10^5 cells (30±8% of control values).

The degree of the epithelial barrier defect was further assessed by determination of the flux of the radiolabelled probe, ^{51}Cr-EDTA. After 48h of co-culture with activated PBM (but not non-activated PBM), the mucosal-to-serosal movement of ^{51}Cr-EDTA across the T84 monolayers was significantly increased compared to control monolayers (3.12±1.49 nmol/h/cm^2 vs 0.76±0.66 nmol/h/cm^2, respectively) (Fig. 2.4).

2.4.3 Epithelial Viability

After 48h, there was no significant difference in LDH released from T84 epithelial cells cultured in media only or co-cultured with activated PBM (1497±71 vs 1560±88 U/L).

2.4.4 Epithelial Physiology after Culture with Conditioned Medium

The altered epithelial ion transport properties (elevation of baseline Isc and reduced responsiveness to forskolin) were also observed following 48h culture with CM from activated PBM (Table2.2). In addition, 48h culture with CM caused a

significant drop in resistance of T84 monolayers compared to control values.

Table 2.2: Effect of A-PBM or conditioned medium on T84 monolayer physiology.

	Physiological properties of T84 monolayers (% of control)		
	Baseline Isc	ΔIsc to FSK	Resistance
A-PBM	251 ± 16*	72 ± 6*	37 ± 4*
A-PBM CM	276 ± 66*	56 ± 2*	49 ± 6*
cA2/A-PBM CM	112 ± 21#	82 ± 3#	82 ± 6#

Confluent T84 monolayers were co-cultured for 48h with LPS/fMLP-activated monocytes (A-PBM) or conditioned medium from activated PBM prepared in the absence (A-PBM CM) or presence of cA2 (cA2/A-PBM CM). Values are mean ± SEM; n=4 experiments; * $p<0.05$ compared to control (100%), # $p<0.05$ compared to A-PBM CM.

2.4..5 The Role of TNFα

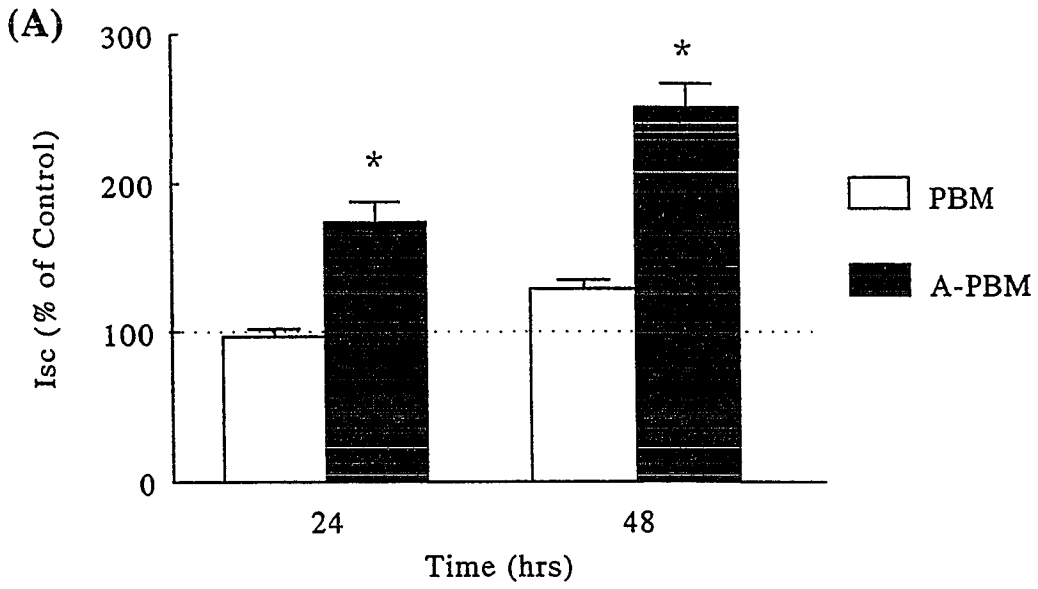
Addition of cA2 (anti-TNFα antibody) to the co-culture system, completely prevented the increase in T84 baseline Isc that had previously been observed following 48h of co-culture with activated PBM (Fig. 2.5). This amelioration of the increased ion transport was accompanied by a return of the active serosal-to-

mucosal Cl^- flux to control levels (0.52±0.05 compared to 0.97±0.11 μEq/h/cm^2 for activated PBM + anti-TNFα vs activated PBM, respectively). In addition, cA2 restored the diminished secretory response to forskolin (92±8% vs 72±6% for activated PBM + anti-TNFα vs activated PBM, respectively) and ameliorated the resistance change and flux of ^{51}Cr-EDTA (Fig. 2.4 and 2.5). Inclusion of the irrelevant antibody had no effect on epithelial barrier and secretory defects.

However, exposure of naive T84 monolayers to 3 or 6 ng/mL of recombinant TNFα (amount measured in CM from activated PBM after 24 and 48h, respectively) for 48h had no significant effect on baseline secretory properties and transepithelial resistance of the epithelial cells (data not shown). In addition, cA2 in CM from activated PBM added to T84 cells did not prove beneficial in correcting the altered epithelial ion secretion (baseline Isc of 276±66% vs 263±57% of control values) or barrier properties (resistance of 49±5.6% vs 48±10% of control values) for CM vs CM + cA2, respectively. This suggested an indirect effect of TNFα, perhaps via autocrine action on PBM themselves. To examine this possibility, T84 monolayers were exposed to the CM which was prepared in the presence of cA2 antibody. Under these conditions, the increased baseline Isc was completely returned to control levels and the reduced T84 resistance was significantly corrected (Table2. 2). This hypothesis was confirmed since T84 monolayers co-cultured with PBM activated with human recombinant TNFα for 48h consistently showed elevated baseline Isc, diminished responses to forskolin and lowered epithelial resistance

which were significantly different from control values (Fig. 2.6). These changes in epithelial function were very similar to those observed in co-culture with LPS/fMLP activated PBM.

Fig. 2.1. Percentage (%) change from control values (T84 cells alone) of epithelial baseline short-circuit current (Isc) after (A) 24 or 48h of co-culture with non-activated monocytes (PBM) or LPS/fMLP-activated monocytes (A-PBM) (n=12 experiments) and (B) following 48h of co-culture with various numbers of A-PBM (n=4 experiments). Values represent mean ± SEM with 2-4 monolayers per experiment; *$p<0.05$ compared to control (100%).


(A)
300
200
100
0
Isc (% of Control)
*
*
PBM
A-PBM
24
48
Time (hrs)

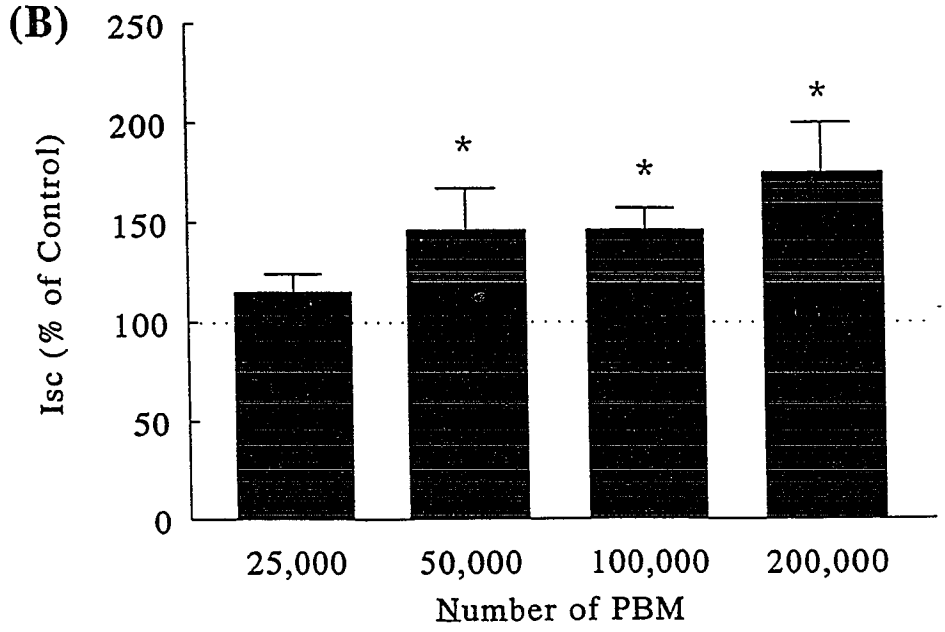

(B)
250
200
150
100
50
0
Isc (% of Control)
*
*
*
25,000
50,000
100,000
200,000
Number of PBM

Fig. 2.2. Percentage (%) changes from control values (T84 cells alone) of epithelial secretory responses to forskolin (10^{-5}M) after 24 or 48h of co-culture with non-activated monocytes (PBM) or LPS/fMLP activated monocytes (A-PBM) (n=12 experiments). Values represent mean ± SEM with 2-4 monolayers per experiment; *$p<0.05$ compared to control (100%).

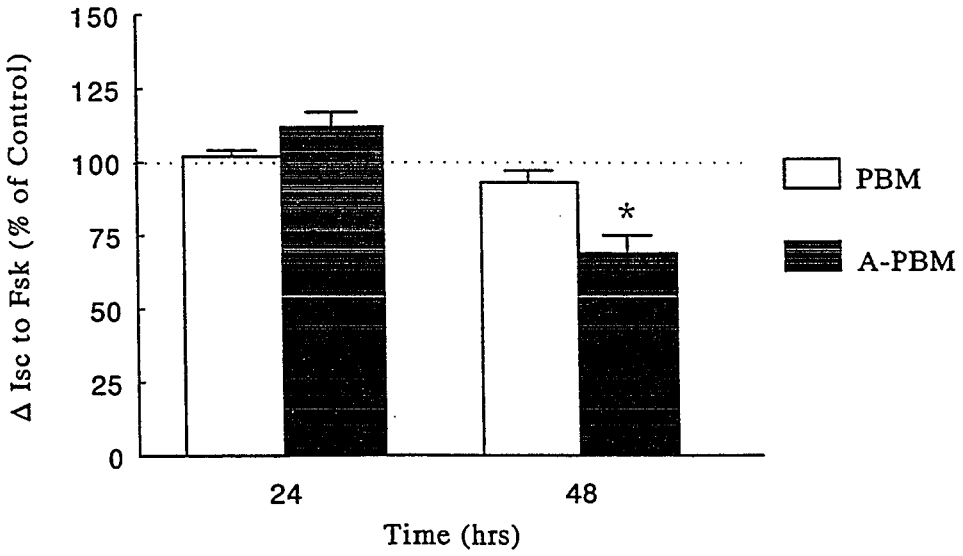
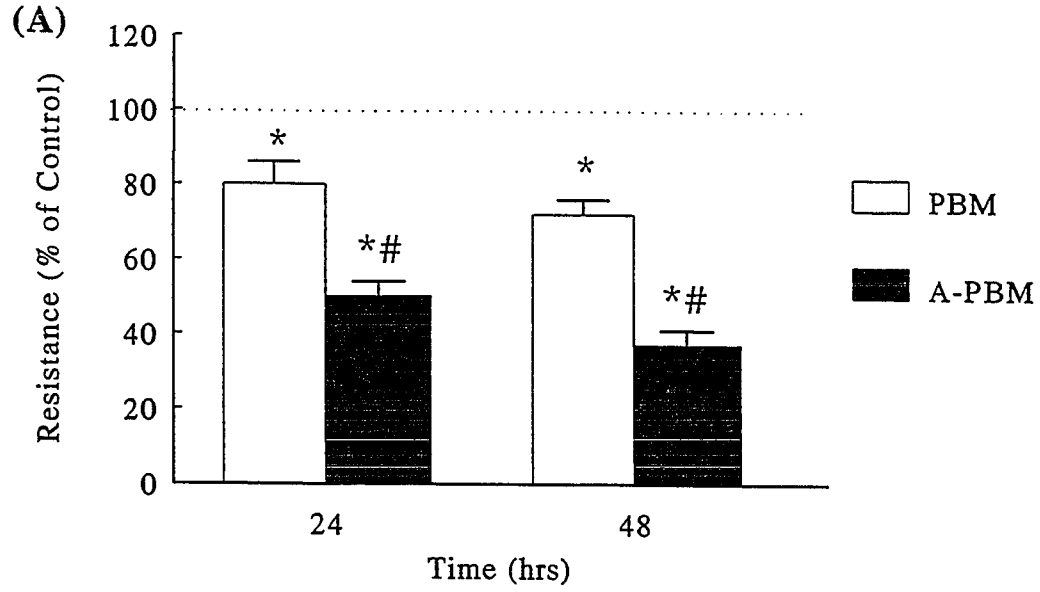

150
125
100
75
50
25
0
Δ Isc to Fsk (% of Control)
24
48
Time (hrs)
*
PBM
A-PBM

Fig.2.3. Percentage (%) change from control values (T84 cells alone) of epithelial resistance after (A) 24 or 48h of co-culture with non-activated monocytes (PBM) or LPS/fMLP-activated monocytes (A-PBM) (n=12 experiments) and (B) following 48h of co-culture with various numbers of A-PBM (n=4 experiments). Values represent mean ± SEM with 2-4 monolayers per experiment; *$p<0.05$ compared to control (100%), #$p<0.05$ compared to PBM.


(A)
120
100
80
60
40
20
0
Resistance (% of Control)
*
*#
*
*#
PBM
A-PBM
24
48
Time (hrs)

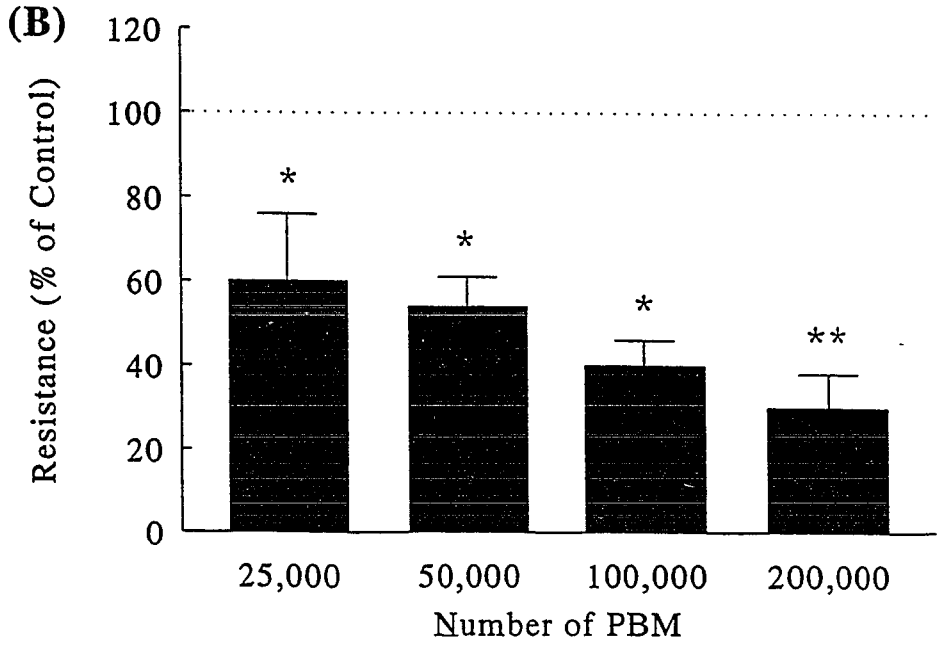

(B)
120
100
80
60
40
20
0
Resistance (% of Control)
*
*
*
**
25,000
50,000
100,000
200,000
Number of PBM

Fig. 2.4. Changes in mucosal to serosal movement of ^{51}Cr-EDTA across T84 monolayers after 48h of co-culture with non-activated monocytes (PBM), LPS/fMLP activated monocytes (A-PBM), or A-PBM with cA2 (1 μg/mL) (n=6 experiments). Values represent mean ± SEM with 2-4 monolayers per experiment; *$p<0.05$ compared to control (100%), #$p<0.05$ compared to A-PBM.

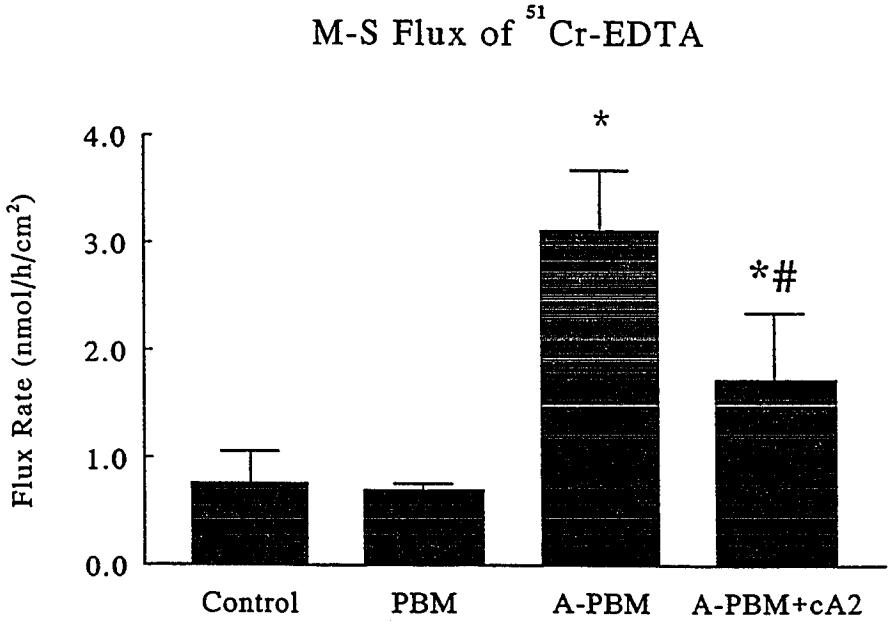

M-S Flux of 51Cr-EDTA
Flux Rate (nmol/h/cm2)
4.0
3.0
2.0
1.0
0.0
*
*#
Control
PBM
A-PBM
A-PBM+cA2

Fig. 2.5. Percentage (%) change from control values (T84 cells alone) of epithelial baseline short-circuit current (Isc) and transepithelial resistance of T84 monolayers following 48h of co-culture with LPS/fMLP activated monocytes (A-PBM) or A-PBM in the presence of cA2 (1μg/mL) (n=8 experiments). Values represent mean ± SEM with 2-4 monolayers per experiment; *$p<0.05$ compared to A-PBM.

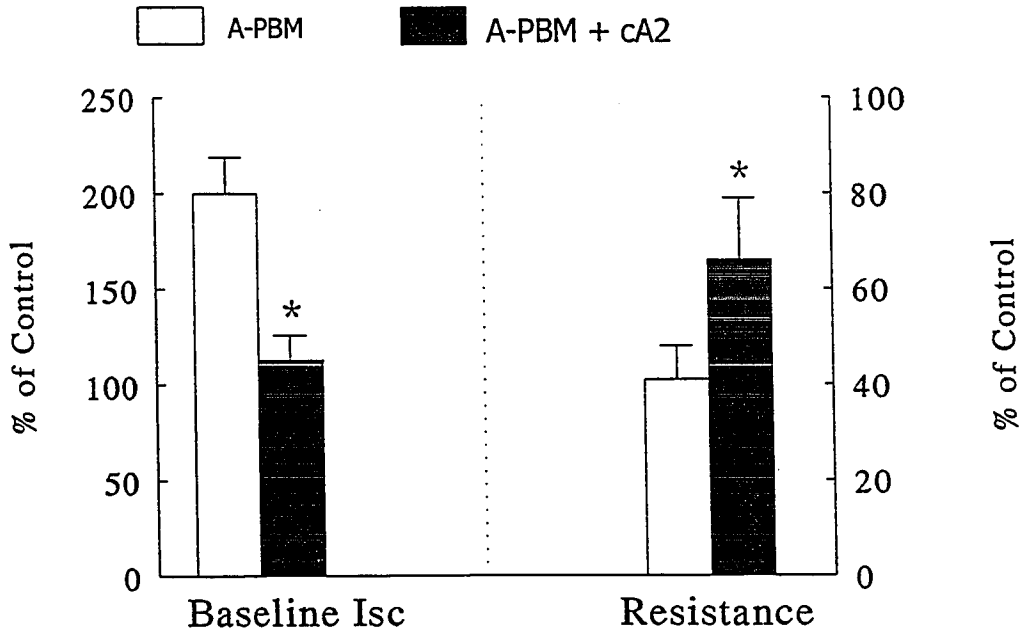
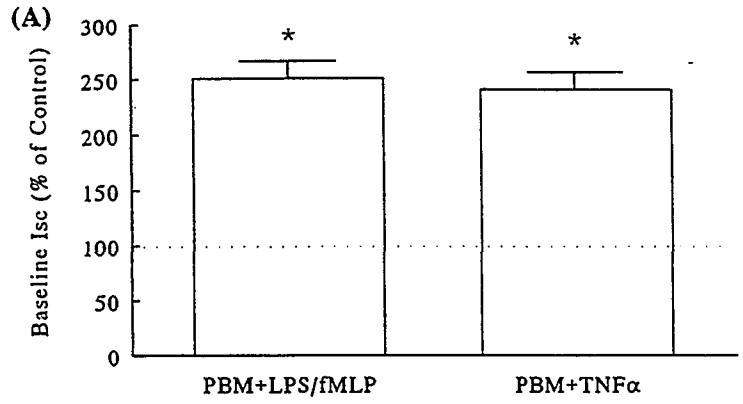
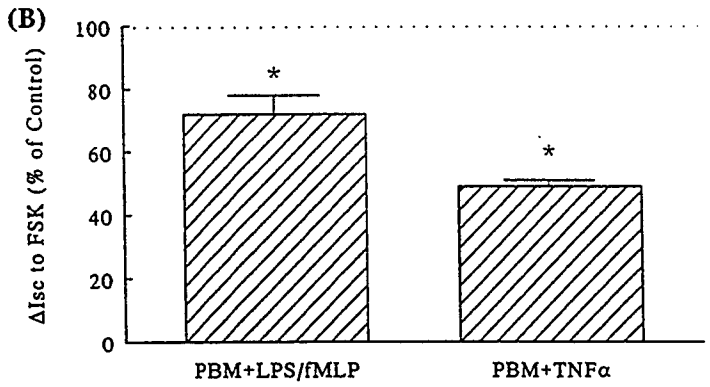

A-PBM
A-PBM + cA2
250
200
150
100
50
0
% of Control
*
*
100
80
60
40
20
0
% of Control
Baseline Isc
Resistance

Fig. 2.6. Percentage (%) change from control values (T84 cells alone) in (A) epithelial baseline short-circuit current (Isc), (B) secretory response to forskolin (10^{-5}M, FSK) and (C) transepithelial resistance of T84 monolayers following 48h of co-culture with LPS/fMLP activated monocytes (PBM + LPS/fMLP) or monocytes activated with human recombinant TNFα (PBM + TNFα; 10 ng/ml) (n=4 experiments). Values represent mean ± SEM with 2-4 monolayers per experiment; *$p<0.05$ compared to control (100%).

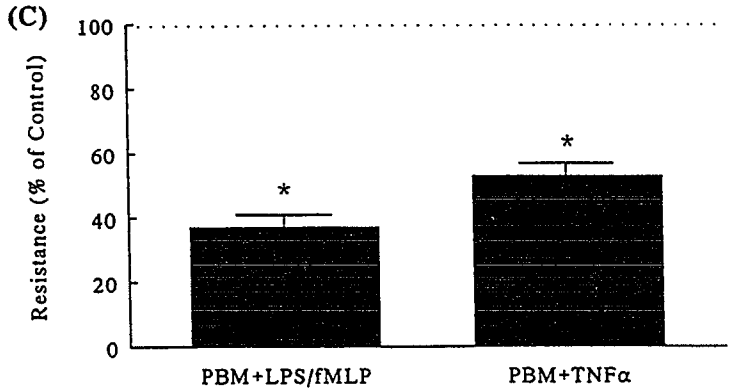
Baseline Isc



ΔIsc to forskolin



Resistance



2.5 DISCUSSION

We have used an *in vitro* co-culture model system to demonstrate that PBM activated with common bacterial products, in the absence of other immune cell types, can alter epithelial ion transport and permeability. Our results showed that after 48h of co-culture, activated PBM stimulated epithelial Cl^- secretion and increased permeability leading to impaired epithelial barrier function. We also demonstrated that PBM-derived TNFα was a key factor in mediating these abnormalities as addition of a neutralizing antibody against TNFα in the co-culture system inhibited the epithelial defects.

Monocytes/macrophages play a central role in immune and inflammatory events in the intestinal mucosa. Resident macrophages are located close to the basal membrane of the intestinal epithelium and represent the first line of defense by immune cells. Additional monocytes may be attracted to the intestinal mucosa during inflammation by locally produced MCP-1 (35). Recent studies have demonstrated the appearance of monocyte subpopulations with a different phenotype in IBD mucosa (22,39) that unlike resident macrophages in normal intestine, express high levels of the LPS receptor, CD14 (4,12,39). The newly recruited cells are more easily activated resulting in the production of excessive amounts of potent inflammatory mediators (2,39). Isolated mononuclear cells from the colonic mucosa of IBD patients have an increased ability to undergo respiratory burst, to stimulate immunoglobulin secretion, and an enhanced antigen presenting

activity (5). Furthermore, it has been recently reported that the $CD14^+$ subset of macrophages from IBD mucosa have a different cytokine profile compared to the resident macrophages and are primed for the production of TNFα, IL-1 and IL-6, all of which can directly or indirectly affect epithelial function (40). Despite this data, the ability of PBM to directly regulate epithelial physiology has not been examined. Additionally, LPS is present in large quantities in the intestinal lumen, and when exposed to LPS, PBM synthesize a plethora of proinflammatory mediators (8,16,27,36) that are capable of inducing local tissue damage through their interactions with T cells, leukocytes and endothelial cells (28). The bacterial tripeptide fMLP (43) induces monocyte chemotaxis and adherence as well as the production of oxygen radicals and pro-inflammatory eicosanoids (26). Since LPS and fMLP are usually present simultaneously in the gut lumen, we chose to add both agents directly to PBM (mimicking events following their uptake from the lumen) and then determine the consequence of this PBM activation on epithelial ion transport and permeability.

Our study clearly shows that PBM activated by LPS/fMLP cause significant changes in epithelial ion transport and barrier functions. Co-culture of T84 cells with activated PBM elicited a significant increase in epithelial ion secretion as shown by the elevation of T84 baseline Isc. The elevated baseline secretion was most pronounced 48h after co-culture and was associated with increased Cl^- secretion. However, the Isc increase was less would be expected based on Cl^-

secretion alone, suggesting that there is another transport event that is decreasing the measured Isc which is not accounted for. From the data presented in Table 1 it appears that altered Na^+ is not responsible. These observations complement findings documenting that LPS-stimulated alveolar macrophages altered ion transport in isolated rat lung epithelial cells (6). In comparison with our findings, T84 monolayers co-cultured with PMNs also display an increase in baseline Isc (19).

Activated PBM increased epithelial ionic permeability as illustrated by a significant reduction in transepithelial resistance of the monolayer after 48h of co-culture. Concomitant with the reduced resistance was an increased transepithelial flux of the inert probe, ^{51}Cr-EDTA, which is suggestive of increased paracellular permeability (34,42). These changes in epithelial permeability were evoked by remarkably few cells (2.5 - 5 x 10^4 PBM). Since epithelial cells were plated at an original density of 10^6 T84 cells/filter, a significant increase in epithelial permeability was observed at a ratio of $\geq$40:1 of epithelial cells to PBM. Similar changes in epithelial physiology have been documented at a ratio of 20:1 of epithelial cells to PMNs (15,19). Thus, our data indicate that PBM have a potent ability to alter epithelial ion transport and permeability. It is clear that PBM can be added to the growing list of immune cells (T cells, neutrophils and eosinophils (19,23,24,37)) that regulate epithelial physiology.

Increased permeability of the epithelial monolayers could potentially be due

to epithelial cell cytotoxicity. However, the effects of activated PBM on T84 cells were not simply the result of decreased epithelial viability, since release of LDH from PBM co-cultured epithelial monolayers was not different from control monolayers. A number of previous studies have shown similar increases in permeability of T84 epithelial monolayers co-cultured with other immune cells (20,24) or infected with enteropathogenic *E.coli* (31) without any significant epithelial cytotoxicity. Moreover, after exposure to activated PBM, the epithelial monolayer was still capable of substantial vectorial ion secretion as indicated by the elevated baseline Isc of the monolayer, significant cAMP- and normal Ca^{2+} - mediated Cl^- secretion. These events indicate a functionally intact monolayer.

Cell-free CM from activated PBM was equally effective in producing the epithelial abnormalities observed after co-culture with activated PBM. Stimulation of PBM by LPS and fMLP leads to the production of an array of proinflammatory cytokines (8,16,27), and among these, TNFα presents itself as a clear candidate for the mediation of the altered epithelial physiology. Several studies have reported increased TNFα protein and mRNA levels in biopsies from IBD patients, particularly in Crohn's disease (17,32). Significantly increased concentrations of TNFα have been reported in stools of children with active chronic IBD (3). Other studies have also reported a rise in circulating TNFα and the soluble TNFα receptor (p55) in patients with active IBD which were significantly correlated with the clinical and/or laboratory measures of disease activity (11,17). Furthermore, in a recent

multicenter, placebo-controlled trial, anti-TNFα antibody (cA2 antibody; same antibody used in this study) treatment resulted in prolonged clinical improvement in some patients with Crohn's disease (46). Therefore, having demonstrated significant TNFα production by PBM to our cocktail of LPS/fMLP, we proceeded to examine the role of TNFα in PBM modulation of epithelial function. Neutralization of TNFα in the co-culture model by addition of an anti-TNFα antibody reduced the T84 baseline Isc response to control levels and significantly improved the T84 transepithelial resistance. In addition, anti-TNFα treatment inhibited the increased epithelial permeability to ^{51}Cr-EDTA evoked by culture with activated PBM. The correction of the abnormal epithelial function by anti-TNFα suggested that TNFα directly affected T84 physiology, alone or in concert with other immune mediators, or that the TNFα effect was one of autocrine activation of PBM. A direct effect of TNFα on HT-29 cells has been demonstrated (38). However, a number of studies have not been able to illustrate a direct action of TNFα on T84 function following an acute exposure (≤72h; 3-6 ng/ml) (20,24,48). In contrast, a recent study has shown a direct effect of TNFα (100 ng/ml) on transepithelial resistance, but only in the presence of IFNγ (47). Another study demonstrated that chronic treatment (4 days) of T84 monolayers with 100 ng/ml of recombinant TNFα caused a significant increase in inulin movement across the T84 monolayers (21).

In exploring the role of TNFα in our model of epithelial disfunction, we found that addition of recombinant TNFα at the concentrations measured in the

supernatant from activated PBM did not affect epithelial physiology. Moreover, neutralization of TNFα in activated PBM CM did not prevent the disrupted secretory responses or the increased epithelial permeability, while inclusion of cA2 at the time of preparation of the CM, resulted in a CM that evoked significantly less epithelial abnormalities. This suggested that the TNFα was autocrinely affecting the PBM, and not the epithelium directly. To further examine this possibility, recombinant TNFα was added to PBM-T84 co-cultures and this resulted in elevated baseline Isc and decreased transepithelial resistance of T84 monolayers. These results were very similar to those evoked by LPS/fMLP activation of PBM. Taken together, these results support the hypothesis that TNFα affected PBM in an autocrine manner (7,45) causing the release of other as yet unidentified monocyte-derived mediators, the net result of which was altered epithelial function. Integrating these findings with previous studies, we suggest that TNFα can modulate epithelial function both directly (studies with HT-29 cells (38)) and indirectly (our study) via immune cell activation.

In summary, we have demonstrated that PBM activated by common bacterial products can stimulate Cl^- secretion, alter ion transport responses and impair the barrier function of the epithelium. We have also shown that TNFα is a key factor mediating the PBM induced pathophysiology, although not affecting T84s directly. It has been suggested that during active IBD, LPS passes through the mucosal barrier, gaining access to PBM and primes them such that subsequent contact with

luminal bacteria results in excessive production of potent inflammatory mediators, particularly TNFα, resulting in pathology/pathophysiology of intestinal tissue (2). Modelling this scenario *in vitro*, we have demonstrated that LPS/fMLP activation of PBM has significant consequences for epithelial ion transport and permeability functions.

The authors thank P. Singh and P. Stetsko for expert technical assistance.

This research was funded in part by Centocor Inc, Astra Draco Pharma and the Crohn's and Colitis Foundation of Canada.

2.6 REFERENCES

1. **Assoian, R. K., B. E. Fleurdelys, and H. C. Stevenson.** Expression and secretion of type β transforming growth factor by activated human macrophages. *Proc. Natl. Acad. Sci. USA* 84: 6020-6024, 1987.

2. **Baldassano, R. N., S. Schreiber, R. B. Johnston, R. D. Fu, T. Muraki, and R. P. MacDermott.** Crohn's disease monocytes are primed for accentuated release of toxic oxygen metabolites. *Gastroenterology* 105: 60-66, 1993.

3. **Braegger, C. D., S. Nicholls, S. H. Murch, S. Stephens, and T. T. MacDonald.** Tumor necrosis factor α in stool as a marker of intestinal inflammation. *Lancet* 339: 89-91, 1992.

4. **Burgio, V. L., S. Fais, M. Boirivant, A. Perrone, and F. Pallone.** Peripheral monocyte and naive T-cell recruitment and activation in Crohn's disease. *Gastroenterology* 109: 1029-1038, 1995.

5. **Cappello, M., S. Keshav, C. Prince, D. P. Jewell, and S. Gordon.** Detection of mRNAs for macrophage products in inflammatory bowel disease by in situ hybridisation. *Gut* 33: 1214-1219, 1992.

6. **Compeau, C. G., O. D. Rotstein, H. Tohda, Y. Marunaka, B. Rafii, A. S. Slutsky, and H. O'brodowich.** Endotoxin-stimulated alveolar macrophages impair lung epithelial Na^+ transport by an L-Arg-dependent mechanism. *Am. J. Physiol.* 266: C1330-C1341, 1994.

7. **Danis, V. A., G. M. Franic, D. A. Rathjen, and P. M. Brooks.** Effects of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, interferon-gamma, tumor necrosis factor-alpha and IL-6 on the production of immunoreactive IL-1 and TNF-α by human monocytes. *Clin. Exp. Immunol.* 85: 143-150, 1991.

8. **Dentener, M. A., V. Bazil, and E. J. U. Von Asmuth.** Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-α, IL-6 and IL-8 release by human monocytes and alveolar macrophages. *J. Immunol.* 150: 2885-2891, 1993.

9. **Dharmsathaphorn, K., J. A. McRoberts, K. G. Mandel, L. D. Tisdale, and H. Masui.** A human colonic tumor cell line that maintains vectorial electrolyte

transport. *Am. J. Physiol.* 246: G204-G208, 1984.

10. **Duchmann, R., I. Kaiser, E. Hermann, W. Mayet, K. Ewe, and K. H. Meyer Zum.** Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin. Exp. Immunol.* 102: 448-455, 1995.

11. **Gardiner, K. R., M. I. Halliday, G. R. Barclay, L. Milne, D. Brown, S. Stephens, R. J. Maxwell, and B. J. Rowlands.** Significance of systemic endotoxaemia in inflammatory bowel disease. *Gut* 36: 897-901, 1995.

12. **Grimm, M. C., P. Pavli, E. Van de Pol, and W. F. Doe.** Evidence for a $CD14^+$ population of monocytes in inflammatory bowel disease mucosa; implications for pathogenesis. *Clin. Exp. Immunol.* 100: 291-297, 1995.

13. **Grimm, M. C., W. E. Pullman, G. M. Bennett, P. J. Sullivan, P. Pavli, and W. F. Doe.** Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. *J. Gastroentero. Hepatol.* 10: 387-395, 1995.

14. **Karnaky, K. J., Jr.** Electrophisiological assessment of epithelia. *Cell-Cell Interactions*, edited by B. R. Stevenson, W. J. Gallin, and D. L. Paul. UK:IRL: Oxford, 1992, p. 257-274.

15. **Kercsmar, C. M., and P. B. Davis.** Resistance of tracheal epithyelial cells to killing by neutrophils, neutrophil elastase and *Pseudomonas* elastase. *Am. J. Respir. Cell Mol. Biol.* 8: 56-62, 1993.

16. **Lord, P. C. W., L. M. G. Wilmoth, and S. B. Mizel.** Expression of interleukin-1α and β genes by human blood polymorphonuclear leukocytes. *J. Clin. Invest.* 87: 1312-1321, 1991.

17. **MacDonald, T. T., P. Hutchings, M. Choy, S. H. Murch, and A. Cooke.** Tumor necrosis factor α and interferon gamma production measured at the single cell level in normal and inflamed human intestine. *Clin. Exp. Immunol.* 81: 301-305, 1990.

18. **Macpherson, A., U. Y. Khoo, I. Forgacs, J. Philpott-Howard, and I. Bjarnason.** Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. *Gut* 38: 365-375, 1996.

19. **Madara, J. L., T. W. Patapoff, B. Gillece-Castro, S. P. Colgan, C. A.**

Parkos, C. Delp-Archer, and R. J. Mrsny. 5'-Adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. *J. Clin. Invest.* 91: 2320-2325, 1993.

20. **Madara, J. L., and J. Stafford.** Interferon-gama directly affects barrier function of cultured intestinal epithelial monolayers. *J. Clin. Invest.* 83: 724-727, 1989.

21. **Madsen, K. L., S. A. Lewis, M. M. Tavernini, J. Hibbard, and R. N. Fedorak.** Interleukin 10 provides cytokine-induced disruption of T84 monolayer barrier integrity and limits chloride secretion. *Gastroenterology* 113: 151-159, 1997.

22. **Mahida, Y. R., S. Patel, P. Gionchetti, D. Vaux, and D. P. Jewell.** Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. *Gut* 30: 826-834, 1989.

23. **Main, C., R. Brattsand, L. Jarkelid, and E. Weislander.** Neutrophil mediated impairment of colonic epithelial cell (Caco-2) barrier function. Effects of protease inhibitors and antioxidants. *Gastroenterology* 106: -A727, 1994.(Abstract)

24. **McKay, D. M., K. Croitoru, and M. H. Perdue.** T cell-monocyte interactions regulate epithelial physiology in a coculture model of inflammation. *Am. J. Physiol* 270: C418-C428, 1996.

25. **Mullin, J. M., and K. V. Snock.** Effect of tumor necrosis factor on epithelial tight junctions and transepithelial permeability. *Cancer Res.* 50: 2172-2176, 1990.

26. **Nast, C. C., and L. E. LeDuc.** Chemotactic peptides: mechanisms, functions, and possible role in inflammatory bowel diseases. *Dig. Dis. Sci.* 33: 50S-57S, 1988.

27. **Nathan, C. F.** Secretory products of macrophages. *J. Clin. Invest* 79: 319-326, 1987.

28. **Nestel, F. P., K. S. Price, T. A. Seemayer, and W. Lapp.** Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor-alpha during graft-versus-host disease. *J. Exp. Med.* 175: 405-413,

1992.

29. **Perdue, M. H., and D. M. McKay.** Integrative immunophysiology in the intestinal mucosa. *Am. J. Physiol.* 267: G151-G165, 1994.

30. **Perdue, M. H., and D. M. McKay.** Mucosal regulators and immunopathophysiology. *Curr. Opin. Gastroentrology* 12: 591-599, 1996.

31. **Philpott, D. J., D. M. McKay, P. M. Sherman, and M. H. Perdue.** Infection of T84 cells with enteropathogenic *Escherchia coli* alters barrier and transport functions. *Am. J. Physiol.* 270: G634-G645, 1996.

32. **Plevy, S. E., C. J. Landers, J. Prehn, N. M. Carramanzana, R. L. Deem, D. Shealy, and S. R. Targan.** A role for TNFα and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. *J. Immunol.* 159: 6276-6282, 1997.

33. **Powell, D. W.** Barrier function of epithelia. *Am. J. Physiol.* 241: G275-G288, 1981.

34. **Ramage, J. K., A. Stanisz, R. Scicchitano, R. H. Hunt, and M. H. Perdue.** Effect of immunological reactions on rat intestinal permeability. Correlation of increased permeability to chromium 51-labled ethylenediaminetetraacetic acid and ovalbumin during acute inflammation and anaphylaxis. *Gastroenterology* 94: 1368-1375, 1988.

35. **Reinecker, H. C., E. Y. Loh, D. J. Ringler, A. Mehta, J. A. Rombeau, and R. P. MacDermott.** Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. *Gastroenterology* 108: 40-50, 1995.

36. **Reinecker, H. C., M. Steffen, T. Witthoeft, I. Pflueger, S. Schreiber, R. P. MacDermott, and A. Raedler.** Enhanced secretion of tumor necrosis factor α, IL-6, and IL-1 β by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clin. Exp. Immunol.* 94: 174-181, 1993.

37. **Resnick, M. B., S. P. Colgan, T. W. Patapoff, R. J. Mrsny, C. S. Awtrey, C. Delp-Archer, P. F. Weller, and J. L. Madara.** Activated eosinophils evoke chloride secretion in model intestinal epithelia primarily via regulated

release of 5'-AMP[1]. *J. Immunol.* 151: 5716-5723, 1993.

38. **Rodriguez, P., M. Heyman, C. Candalh, M. A. Blaton, and C. Bouchaud.** Tumor necrosis factor-α induces morphological and functional alterations of intestinal HT29 cl.19A cell monolayers. *Cytokine* 7: 441-448, 1995.

39. **Rugtveit, J., P. Brandtzaeg, T. S. Halstensen, O. Fausa, and H. Scott.** Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes. *Gut* 35: 669-674, 1994.

40. **Rugtveit, J., E. M. Nilsen, A. Bakka, H. Carlsen, P. Brandtzaeg, and H. Scott.** Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. *Gastroenterology* 112: 1493-1505, 1997.

41. **Sartor, R. B.** Role of intestinal microflora in initiation and prepetuation of inflammatory bowel disease. *Can. J. Gastroenterol.* 4: 271-277, 1990.

42. **Saunders, P. R., U. Kosecka, D. M. McKay, and M. H. Perdue.** Acute stressors stimulate ion transport and increase epithelial permeability in the rat intestine. *Am. J. Physiol.* 267: G794-G799, 1994.

43. **Sherriff, R. M., M. F. Broom, and V. S. Chadwick.** Isolation and purification of N-formylmethionine aminopeptidase from rat intestine. *Biochem. Biophys. Acta* 1119: 275-280, 1992.

44. **Surette, M. E., R. Palmantier, and J. Gosselin.** Lipopolysaccharides prime whole human blood and isolated neutrophils for the increased synthesis of 5-lipoxugenase products by enhancing arachidonic acid availability-involvment of the CD14 antigen. *J. Exp. Med.* 178: 1347-1355, 1993.

45. **Sweet, M. J., and D. A. Hume.** Endotoxin signal transduction in macrophages. *J. Leukoc. Biol.* 60: 8-26, 1996.

46. **Targan, S. R., S. B. Hanauer, S. J. H. Van Deventer, L. Mayer, D. H. Present, T. A. J. Braakman, K. L. DeWoody, T. F. Schaible, and P. Rutgeerts.** A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor α for crohn's disease. *N Engl J Med* 337: 1029-1035, 1997.

47. **Taylor, C. T., A. L. Dzus, and S. P. Colgan.** Autocrine regulation of epithelial permeability by hypoxia: role for polarized release of tumor necrosis factor α. *Gastroenterology* 114: 657-668, 1998.

48. **Taylor, C. T., A. Murphy, D. Kelleher, and A. W. Baird.** Changes in barrier function of a model intestinal epithelium by intraepithelial lymphocytes require new protein synthesis by epithelial cells. *Gut* 40: 634-640, 1997.

49. **Zund, G., J. L. Madara, A. L. Dzus, C. S. Awtrey, and S. P. Colgan.** Interleukin-4 and interleukin-13 differentially regulate epithelial chloride secretion. *J. Biol. Chem.* 271: 7460-7464, 1996.

CHAPTER 3

MONOCYTE/MACROPHAGE ACTIVATION BY NORMAL BACTERIA AND BACTERIAL PRODUCTS: IMPLICATIONS FOR EPITHELIAL PATHOPHYSIOLOGY IN CROHN'S DISEASE

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American Journal of Pathology (Submitted)

3.1 ABSTRACT

Intestinal immune cells are less reactive than those in the circulation; however, such cells from patients with Crohn's disease may be more responsive to bacterial products. Our study examined if non-pathogenic bacteria or lipopolysaccharide (LPS), can induce epithelial pathophysiology in the presence of monocytes/macrophages. Lamina propria mononuclear cells (LPMC) and peripheral blood monocytes (PBM) were obtained from patients with Crohn's disease and control subjects. Filter grown T84 epithelial monolayers were co-cultured with non-activated or LPS-activated LPMC or PBM for 48h. Epithelial secretory (baseline short-circuit current [Isc] and ΔIsc to 10^{-5}M forskolin) and barrier (transepithelial electrical resistance [TER]) parameters were measured in Ussing chambers. LPS-activated PBM from both controls and patients with Crohn's disease significantly increased Isc (~300%) and reduced TER (~40%). No pathophysiology occurred after co-culture with control LPMC ± LPS. However, non-activated Crohn's disease LPMC spontaneously secreted tumor necrosis factor α (TNFα), and induced changes similar to those produced by activated PBM. Control *Escherichia coli*+ PBM induced comparable functional changes in epithelial physiology, which were abrogated by TNFα antibody. We conclude that the intestinal macrophages of patients with Crohn's disease are spontaneously activated, possibly by Gram negative luminal bacteria, and can directly cause epithelial pathophysiology.

3.2 INTRODUCTION

Crohn's disease is a chronic inflammatory bowel disease (IBD) that can affect any part of the gastrointestinal tract. The precise nature of the initiating events involved in Crohn's disease are not known, although findings in various animal models [1-3] and human studies [4-6] support the concept that either an inappropriate or exaggerated immune response is responsible for much of the pathogenesis of the disease. This immune response may be driven by exposure to enteric bacteria (commensal or pathogenic) or bacterial products such as LPS.

Recently, attention has focused on cells of the monocyte/macrophage lineage and their inflammatory products. These phagocytic cells are scattered beneath the intestinal epithelium and represent the first line of defense against foreign antigens. Previous studies indicate that inflammatory conditions of the gut are associated with increased heterogeneity and activation of intestinal macrophages. There is a distinct population of recently recruited monocyte-like macrophages in actively inflamed IBD lesions [7,8]. These cells are more reactive than resident macrophages, spontaneously releasing bioactive products and cytokines such as TNFα [9]. TNFα has recently become the target of clinical investigations in studies inhibiting its effects using a human-murine chimeric monoclonal antibody, Remicade™ (previously known as cA2; Centocor Inc., Malvern, PA)[10-12].

The intestinal epithelium is a dynamic barrier which regulates absorption of

nutrients and water and at the same time restricts uptake of microbes and other noxious material from the gut lumen. One of the distinct features of Crohn's disease is impaired intestinal epithelial physiology, characterized by increased permeability (altered barrier function) and ion secretion creating a luminally directed driving force for water movement which can result in diarrhea. Indeed in a previous study we showed that co-culture of a model epithelium with LPS-activated normal monocytes resulted in epithelial abnormalities reminiscent of those observed in resected tissue from IBD.

Despite increasing evidence of an association between the commensal bacterial microflora and intestinal inflammation[13-15], little information is currently available on the direct role of commensal bacteria in altering gut epithelial function. This is partly due to the lack of a convenient experimental model to explore the interactions of normal flora with mucosal immune cells (particularly macrophages), in the regulation of intestinal epithelial physiology. We recently described a co-culture model of epithelial cells and immune cells[16] which was adapted for the current studies. The aim of the present study was two fold: 1) to compare the ability of peripheral blood monocytes (PBM) and lamina propria mononuclear cells (LPMC) from patients with Crohn's disease and control subjects ± LPS activation to affect enteric epithelial ion transport and barrier functions; and 2) to assess the ability of a luminally applied non-pathogenic (≡commensal) strain of bacteria to modulate epithelial function directly and in the context of monocyte/macrophages in a sub-

epithelial position. Our objective was to gain further knowledge regarding the role of commensal microorganisms and the responsiveness of gut derived immune cells to bacterial products in affecting epithelial function in IBD.

3.3 MATERIALS AND METHODS

3.3.1 Isolation of peripheral blood monocytes (PBM)

Peripheral blood was collected from ambulatory patients with Crohn's disease attending the IBD clinic at McMaster University Medical Center. Blood samples from healthy volunteers were obtained for control experiments. Whole blood was diluted in phosphate-buffered saline (PBS) and subjected to one-step density centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). The interface containing mononuclear cells was carefully collected, washed in PBS and resuspended in fresh T84 culture medium (1:1 mixture of DMEM and F-12 medium supplemented with 1.5% HEPES, 2% penicillin-streptomycin, and 10% FCS) at 10^6 cells/ml[16]. The PBM population was obtained by plastic plating of peripheral blood mononuclear cells (4h at 37°C) and subsequent removal of non-adherent T and B cells. Fresh medium was added to the adherent cells, which were then incubated for 18 h at 37°C prior to use in co-culture studies.

3.3.2 Isolation of lamina propria mononuclear cells (LPMC)

Surgical specimens of inflamed small or large intestine were obtained from patients undergoing resection for non-fibrotic Crohn's disease. Uninflammed control small or large bowel was obtained from patients undergoing surgery for cancer, at least 5 cm from the tumor margin. LPMC were isolated by a modification

of the technique described by Bull and Bookman[17]. Briefly, surgical specimens were washed extensively in RPMI 1640 (GIBCO laboratories, Grand Island, NY) containing 1 mM dithiothreitol (DTT; Sigma Chemical Co., St. Louis, MO) and 5% fetal calf serum (FCS;GIBCO) and mucosa dissected free from the muscle layer. It was then cut into small pieces, washed, and incubated for 20 minutes in RPMI containing DTT at 37°C. Epithelial cells were then removed by two 30 min incubations in Hanks balanced salt solution (GIBCO) containing 1mM ethylenediaminetetraacetic acid (EDTA; Sigma) and 10% FCS at 37°C. The mucosal samples were washed and incubated overnight with gentle stirring in RPMI 1640 containing 1 U/ml collagenase (Sigma), 6 U/ml DNase II (Sigma) and 10% FCS. The tissue digest was then washed and filtered through a 200 μm steel mesh. The cells were washed twice in RPMI 1640 containing 10% FCS and subjected to Percol density gradient centrifugation. The interface was carefully removed, washed and resuspended in T84 culture medium at a concentration of 10^6 cells /ml. LPMC were incubated for 18h at 37°C before use in co-culture studies.

The percentage of macrophages in LPMC preparations was determined by differential staining (HEMA-TEK 2000 slide stainer; MILES Inc. Diagnostic Division, Eikhart, IN) to be 7-15% of the total cell population for both normal and Crohn's disease.

3.3.3 Epithelial cells

Human T84 colonic epithelial cells (passage 45-60) were seeded onto tissue culture-treated semi-permeable filter supports (0.4 μm pore size, 1.0 cm^2 surface area; Costar Corporation, Cambridge, MA) at a concentration of 10^6 cells/mL and grown in T84 culture medium. Cells were grown at 37°C for 7 days to form polarized epithelial monolayers, displaying transepithelial electrical resistances of greater than 1,000 $\Omega.cm^2$ as measured by voltmeter (Millicell-RES; Millipore Corporation, Bedford, MA).

3.3.4 Bacterial strain and growth conditions

Bacteria of the non-pathogenic, laboratory control strain of *Escherichia coli*, HB101[18,19] were grown in nonaerated Trypticase soy broth for 6h at 37°C. Bacteria were pelletted by centrifugation at 2,400 g for 15 minutes and then resuspended in sterile antibiotic-free T84 culture medium to a concentration of approximately 1 x 10^9 colony forming units (CFU)/ml. Viable bacteria were enumerated by serial 10-fold dilutions plated onto horse-blood agar plates.

3.3.5 Co-culture studies

Culture of immune cells with epithelial cells

Experiments with PBM: PBM were activated by addition of 10 μg/ml lipopolysaccharide (LPS) *(Escherichia coli* O111:B4, O127:B8, or *Salmonella*

minnesota; Sigma) to the culture medium at the time of co-culture. Confluent T84 monolayers were co-cultured for 48h with normal or Crohn's disease PBM (approximately 1.5 x 10^5 cells/well) with or without LPS placed into the basal compartment of the culture wells. Controls were naive T84 monolayers cultured at the same time but in the absence of immune cells or with LPS alone. The functional properties of the monolayers were the same in both control groups, so naive monolayers were considered as standard controls.

Experiments with LPMC: Preliminary experiments demonstrated that similar results were obtained when T84 monolayers were co-cultured with freshly isolated LPMC + LPS or with 24 h supernatants from LPS-activated LPMC. Therefore, in some experiments supernatants were prepared and stored frozen at -70°C until several were collected. Then experiments were conducted for 48 h by adding supernatants to cultures of confluent T84 monolayers. Controls were the same as those for the PBM experiments.

Addition of non-pathogenic E. coli

Confluent T84 monolayers were co-cultured for 48h with PBM in the basal compartment, and 10^5 *E. coli* strain HB101 added to the apical compartment (luminal side) of the transwells. Controls were naive T84 monolayers or those exposed to HB101 alone.

3.3.6 Ussing chamber experiments

Epithelial ion transport

Following culture with cells or supernatants, T84 monolayers on filter supports were mounted into Ussing chambers, as previously described[16]. Briefly, monolayers were bathed in oxygenated Krebs buffer (pH 7.35, 37°C), containing 115 mM NaCl, 8 mM KCl, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 2.0 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose as an energy source in the basal compartment. In the apical compartment, 10 mM mannitol was substituted for glucose. The epithelial spontaneous potential difference (PD, in mV) was maintained at zero volts by the continuous injection of an external current by an automated voltage clamp (World Precision Instruments Inc, Sarasota, FL). This short-circuit current (Isc, in $\mu A/cm^2$) reflects net active ion transport across the preparation. Baseline Isc was recorded after a 15 min equilibration period. Stimulated ion secretion was measured by adding the adenylate cyclase-activating agent, forskolin (10^{-5} M) (Sigma), to the serosal side of the T84 monolayers and recording the maximum increase in Isc.

Epithelial barrier function

Electrical resistance is a measure of the barrier function of the epithelium to passive ion movement. At intervals during each experiment, PD across the monolayer was clamped at 1.0 mV (differential pulse method, 1 pulse/30 seconds). The resulting change in current was measured and the transepithelial electrical

resistance (TER, in Ω/cm^2) was calculated according to Ohm's law.

As an indication of epithelial permeability to larger molecules, in experiments conducted with normal PBM (± *E. coli*) the mucosal to serosal movement of the inert probe, ^{51}Cr-EDTA (362.3 Da, diameter 1.15 nm) was measured by adding ^{51}Cr-EDTA (6.5 µCi/mL) (Radiopharmacy, McMaster-Chedoke Hospital, Hamilton, ON, Canada) to the mucosal buffer and non-radioactive Cr-EDTA to the serosal buffer to maintain the osmotic balance. Fluxes were determined at 30 min intervals.

3.3.7 Tumor necrosis factor α (TNFα)

Basal or stimulated production of the proinflammatory cytokine, TNFα, by PBM and LPMC was measured at 24h by ELISA with a sensitivity of 7.4 pg/ml (TNFα-FLEXIA; BioSource International, Camarillo, CA).

The role of TNFα in *E. coli*-PBM modulation of epithelial ion transport and barrier functions was assessed by inclusion of a neutralizing antibody to TNFα, cA2 (1 µg/ml, >100 fold excess of the TNFα measured in the conditioned medium (CM); Centocor Inc., Malvern, PA). An irrelevant isotype matched antibody (anti-hepatoma IgG1, AF20; Centocor) was used as a control.

3.3.8 Statistics

Results are presented as mean ± SEM. Due to variability in absolute values between different batches of T84 cells, data were normalized to control values in

each experiment (expressed as percentage of control); n values represent the number of experiments (different blood donors or tissue samples) in which 2-4 monolayers were examined for each condition. Data were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls comparison. Student's t test was used where appropriate for individual comparisons. Statistically significant differences were accepted at $p < 0.05$.

3.4 RESULTS

3.4.1 Effects of PBM (± LPS) on epithelial physiology

Epithelial ion transport

Control T84 epithelial monolayers displayed a baseline Isc of 2.2 ± 0.4 $\mu A/cm^2$ which was not significantly altered after 48 h co-culture with non-activated PBM or LPS alone. However, co-culture with PBM activated by LPS significantly ($P<0.05$) elevated the baseline Isc of the T84 monolayers to 251 ± 16% of control values, as demonstrated in Fig. 3.1A. Similar results were obtained when T84 monolayers were co-cultured with PBM from patients with Crohn's disease. No change in baseline Isc was observed following co-culture of T84 cells with non-activated Crohn's disease PBM, but LPS addition did evoke a significant ($P < 0.05$) increase in baseline Isc to 296 ± 58% of control values and a diminished Isc response to forskolin (ΔIsc) (76 ± 6% of control values, $P < 0.05$). This was comparable to the changes induced by LPS-activated normal PBM (69 ± 6% of control values) (Fig. 3.1B).

Epithelial barrier function

Control T84 monolayers displayed a TER of 1,784 ± 179 Ω/cm^2. Changes in TER of T84 monolayers after co-culture with PBM ± LPS are demonstrated in Fig. 3.1C. Co-culture with non-activated PBM produced a small drop in TER (to 78 ±

6% and 75 ± 10% of control values; normal PBM and Crohn's disease PBM, respectively). This change in the epithelial barrier function was enhanced following activation of PBM by exposure to LPS. After 48h of co-culture with activated PBM, T84 resistance was significantly (P<0.005) reduced to 37 ± 5% and 49 ± 7% of control values (normal PBM and Crohn's disease PBM, respectively).

TNFα production by PBM

Non-activated normal PBM produced no detectable TNFα (limit of detection was 7.4pg/ml). LPS activation of PBM caused a significant increase in TNFα secretion after 24h (mean: 2332 pg/ml; range: 346-4595 pg/ml; n=10). Similar to normal PBM, PBM isolated from Crohn's disease patients' blood did not secrete TNFα under basal conditions, but did demonstrate TNFα production when activated by LPS (mean: 1921 pg/ml; range: 590-3304 pg/ml; n=6) (Fig. 3.2A).

3.4.2 Effects of LPMC (± LPS) on epithelial physiology

The results obtained from small and large bowel LPMC were comparable for all parameters measured and therefore are combined for presentation.

Epithelial ion transport

The baseline Isc of T84 monolayers was unchanged following co-culture with LPMC isolated from normal mucosa (Fig. 3.3A) and remained comparable to control

levels after adding LPS to the co-culture (86 ± 6% and 102 ± 16% of control values, normal LPMC and normal LPMC + LPS, respectively). In contrast, LPMC isolated from Crohn's disease mucosa spontaneously evoked an elevated baseline Isc of T84 monolayers after 48h of co-culture (198 ± 40% of control values, $P<0.05$). LPS addition produced no further increase in the baseline Isc of T84 cell monolayers (188 ± 21% of control values).

As shown in Fig. 3.3B, the ΔIsc increase evoked by forskolin was unaltered by co-culture with normal LPMC (94 ± 8% of control) even in the presence of LPS (98 ± 10% of control). However, there was a significantly diminished response to forskolin after co-culture with LPMC from Crohn's disease mucosa, and inclusion of LPS resulted in no additional effects on T84 responses (70 ± 8% and 72 ± 8% of control values; Crohn's disease LPMC and Crohn's disease LPMC + LPS, respectively).

Epithelial barrier function

T84 monolayers co-cultured with normal LPMC, in the absence or presence of LPS, displayed TER that was not different from control monolayers (Fig. 3.3C). In contrast, co-culture with LPMC from Crohn's disease mucosa, led to a decrease in TER of T84 cells (34 ± 9% of control values, $P<0.005$) with no further reduction when LPS was added (28 ± 5% of control values).

TNFα production by LPMC

The spontaneous secretion of TNFα by LPMC isolated from normal mucosa was below the detection limit (<7.4 pg/ml) for all samples (n=6) and stimulation by LPS had no effect on TNFα secretion. In contrast, the spontaneous secretion of TNFα by LPMC obtained from Crohn's disease tissue was markedly elevated (mean: 168 pg/ml; range: 29-236 pg/ml; n=8) compared to normal LPMC and was further stimulated by LPS to approximately 2 fold (mean: 354 pg/ml; range: 76-590 pg/ml) (Fig. 3.2B).

3.4.3 Comparison of LPS-activated PBM and non-activated LPMC from Crohn's disease

As illustrated in Table 3.1, striking similarities were found between the T84 functional changes induced by Crohn's disease LPMC in the absence of LPS and those induced by LPS-activated PBM. These data suggest that macrophages in the mucosa of patients with Crohn's disease are spontaneously activated, possibly by bacteria or their products, and directly influence intestinal epithelial physiology in a manner similar to LPS-activated circulating PBM. Therefore, we utilized normal PBM to examine if a non-pathogenic control strain of *E. coli* (HB101), added to the luminal compartment (physiological side) of T84 cell monolayers, could induce epithelial dysfunction.

Table-3.1. Comparison of epithelial pathophysiology induced by LPS-activated PBM and spontaneously-activated LPMC from Crohn's disease

	Properties of T84 Monolayers (% of control)		
	Baseline Isc	ΔIsc to Fsk	TER
PBM (+LPS)	251 ± 16*	69 ± 6*	37 ± 5**
Crohn's LPMC	198 ± 40*	70 ± 8*	34 ± 9**

Values are means ± SEM of 10-14 experiments. Confluent T84 monolayers were co-cultured for 48 h with peripheral blood monocytes (PBM) + LPS (10μg/ml), or spontaneously activated lamina propria mononuclear cells (LPMC) from Crohn's mucosa; Fsk, forskolin (10^{-5} M). *P<0.05 and **P<0.005 compared to control (T84 monolayers alone) at 100%.

3.4.4 Effects of PBM and luminal bacteria on epithelial physiology

Epithelial ion transport

The baseline Isc of T84 monolayers was unchanged after co-culture with luminal control *E. coli* alone, with values comparable to controls (98 ± 4% of control values) (Fig. 3.4A). However, co-culture with luminal bacteria in the presence of basal PBM resulted in a significantly (P<0.05) elevated baseline Isc of the T84

monolayers (217 ± 39% of control values). As demonstrated in Fig. 3.4B, the ΔIsc evoked by forskolin remained unchanged after co-culture with luminal control *E. coli* alone (93 ± 11 % of control values) but significantly ($P<0.05$) diminished in the presence of basal PBM (61 ± 7% of control values).

Epithelial barrier function

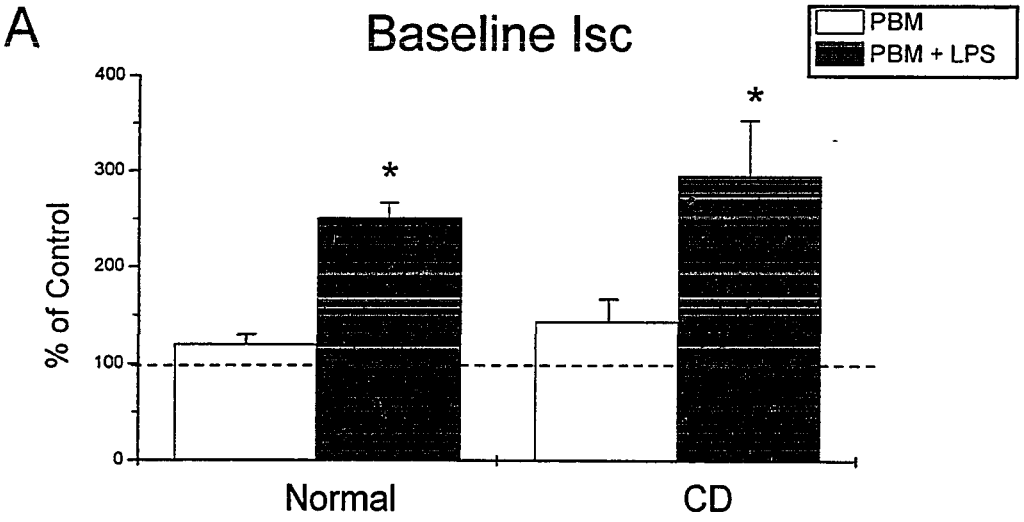
Co-culture of the T84 monolayers with luminal control *E. coli* alone had no effect on the TER. However, the resistance of the monolayers was reduced (54 ± 7% of control values; $P<0.05$) following 48 h co-culture in the presence of PBM (Fig. 3.5A). The degree of the epithelial barrier defect was further assessed by determination of the flux of ^{51}Cr-EDTA. After 48 h co-culture with bacteria and PBM (but not bacteria alone), the serosal to mucosal flux of ^{51}Cr-EDTA across T84 monolayers was significantly ($P<0.05$) elevated (3.9 ± 0.8 nmol.h^{-1}.cm^{-2}) compared with control monolayers (0.9 ± 0.2 nmol.h^{-1}.cm^{-2}) (Fig. 3.5B). This increase in epithelial permeability is in agreement with our previous study, in which normal PBM activated by bacterial products induced an increase in flux of ^{51}Cr-EDTA[16].

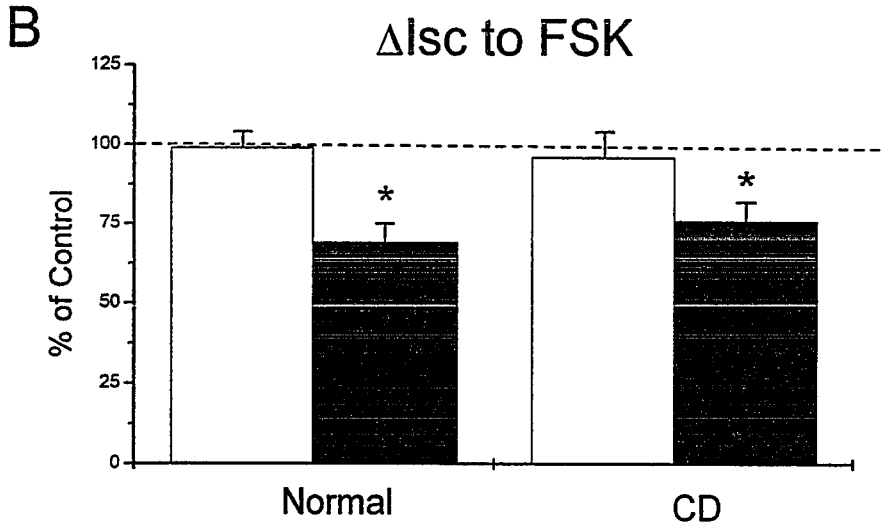
TNFα production and effect of anti-TNFα treatment

PBM stimulated indirectly by co-culture with the non-pathogenic *E. coli* secreted substantial amounts of TNFα after 24 h (mean: 2240 pg/ml; range 984-2907 pg/ml; n=5). Addition of anti-TNFα antibody at the start of the co-culture

completely prevented the increase in baseline Isc of the T84 monolayers (to 113 ± 19% of control values), and restored the diminished ΔIsc response to forskolin (to 98 ± 12% of control values). In addition, anti-TNFα ameliorated both the change in the TER and the increased flux of ^{51}Cr-EDTA of the epithelial monolayers, caused by control *E. coli* in the presence of PBM (Figs. 3.4A and B, 3.5A and B). By contrast, inclusion of the irrelevant antibody had no effect on epithelial secretory or barrier defects (data not shown).

Fig. 3.1. Percent changes from control values (T84 cells alone) in epithelial baseline Isc (A), ΔIsc in response to forskolin (FSK) (B), and transepithelial resistance (C) of T84 monolayers after 48h of co-culture with normal or Crohn's disease (CD) peripheral blood monocytes (PBM) in the presence or absence of LPS (n=12-14 donors). Values represent mean ± SEM with 2-4 monolayers per condition. *$P<0.05$ and **$P<0.005$ compared to control (100%).


A
Baseline Isc
PBM
PBM + LPS
% of Control
400
300
200
100
0
*
*
Normal
CD


B
ΔIsc to FSK
% of Control
125
100
75
50
25
0
*
*
Normal
CD

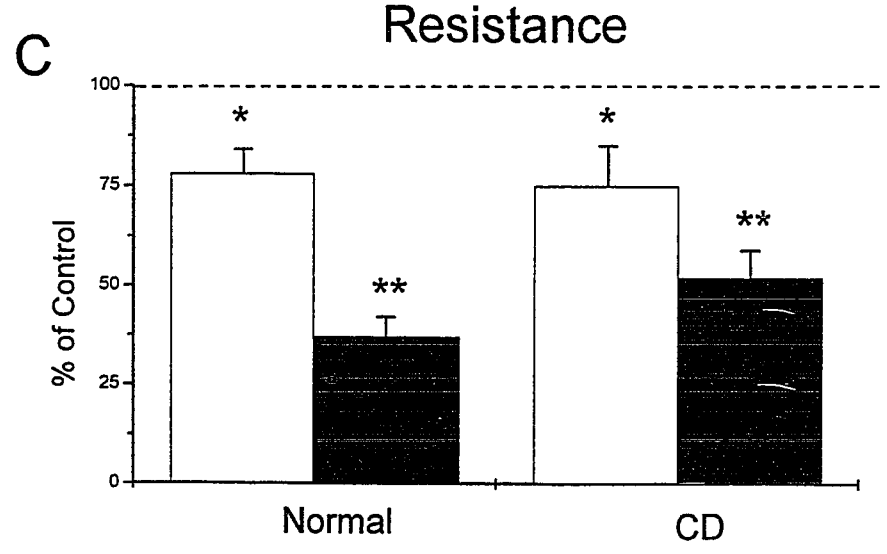
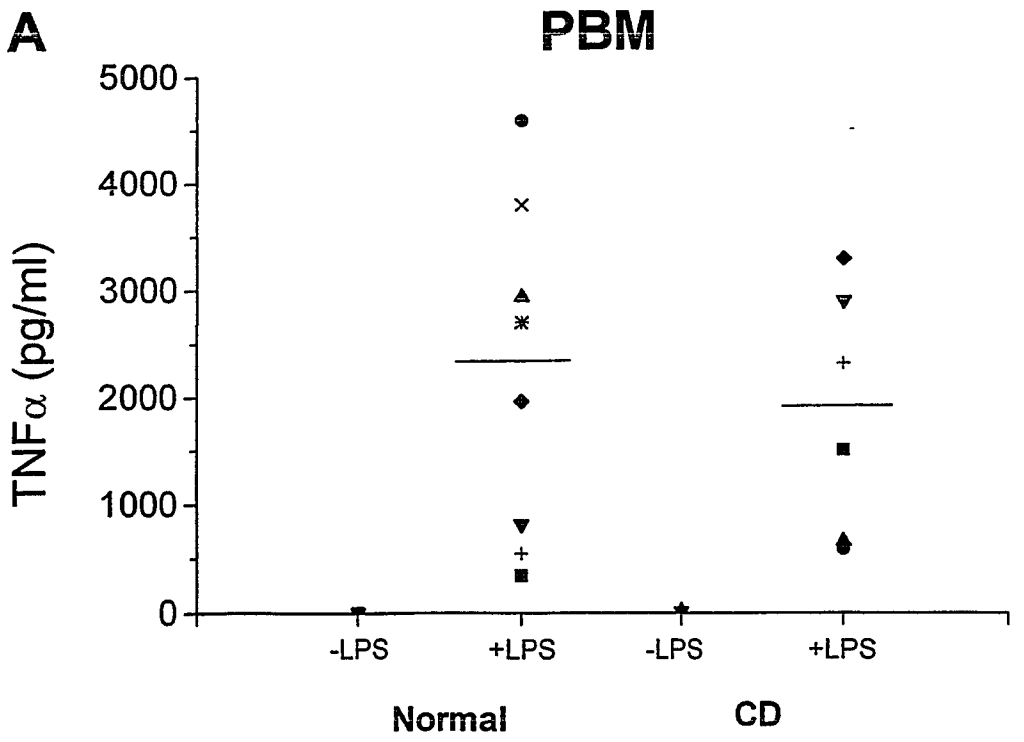

C
Resistance
% of Control
100
75
50
25
0
*
*
**
**
Normal
CD

Fig. 3.2. Basal (-LPS) or stimulated (+LPS) TNFα production by peripheral blood monocytes (PBM) (A) and lamina propria mononuclear cells (LPMC) (B) from normals or patients with Crohn's disease (CD) after 24 h of co-culture (n=6-8 donors).


A
PBM
5000
4000
3000
2000
1000
0
TNFα (pg/ml)
-LPS
+LPS
-LPS
+LPS
Normal
CD

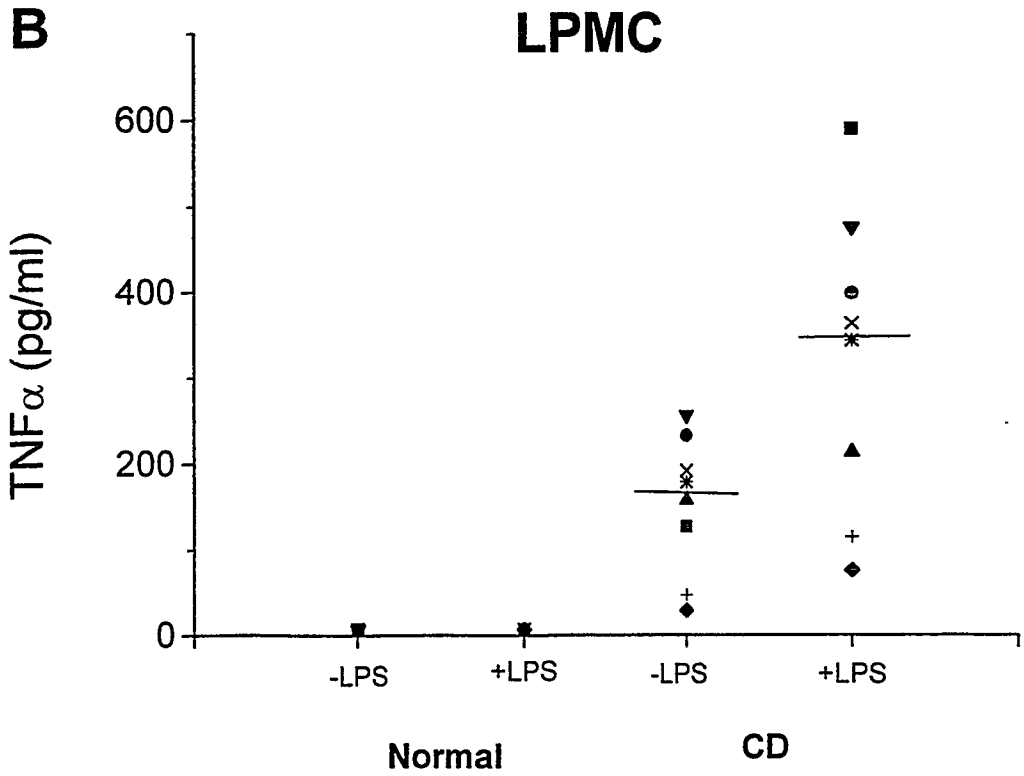
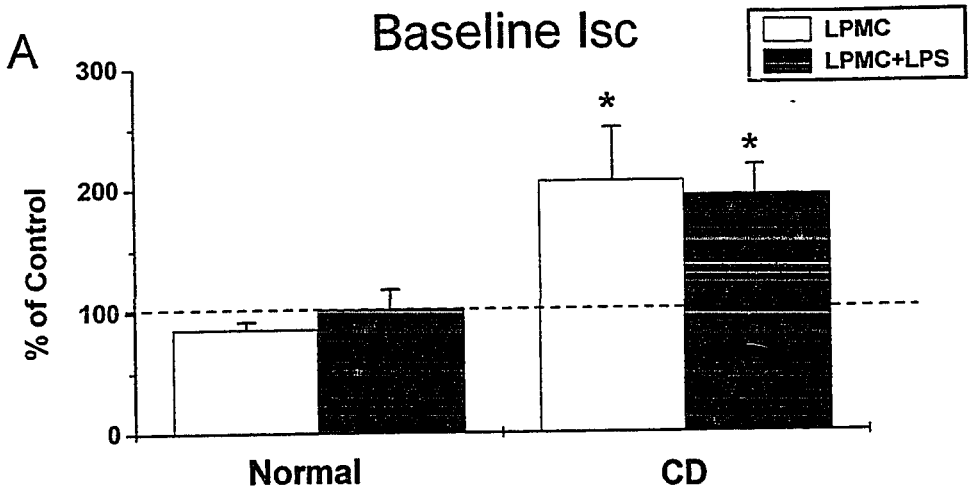
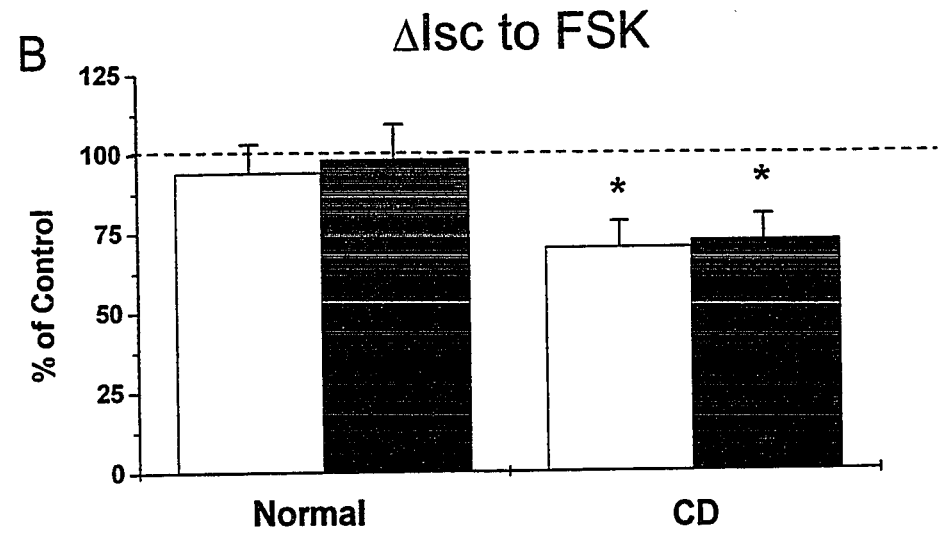

B
LPMC
600
400
200
0
TNFα (pg/ml)
-LPS
+LPS
-LPS
+LPS
Normal
CD

Fig. 3.3. Percent changes from control values (T84 cells alone) in epithelial baseline Isc (A), ΔIsc in response to forskolin (FSK) (B), and transepithelial resistance (C) of T84 monolayers after 48h of co-culture with normal or Crohn's disease (CD) lamina propria mononuclear cells (LPMC) in the presence or absence of LPS (n=10-12 specimens). Values represent mean ± SEM with 2-4 monolayers per condition. *P<0.05 and **P<0.005 compared to control (100%).


A
Baseline Isc
LPMC
LPMC+LPS
% of Control
300
200
100
0
*
*
Normal
CD


B
ΔIsc to FSK
% of Control
125
100
75
50
25
0
*
*
Normal
CD

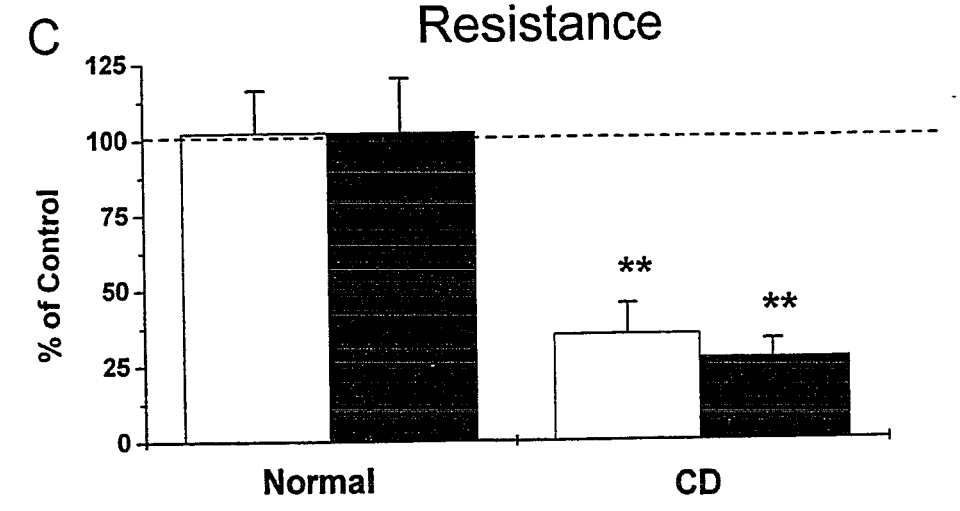
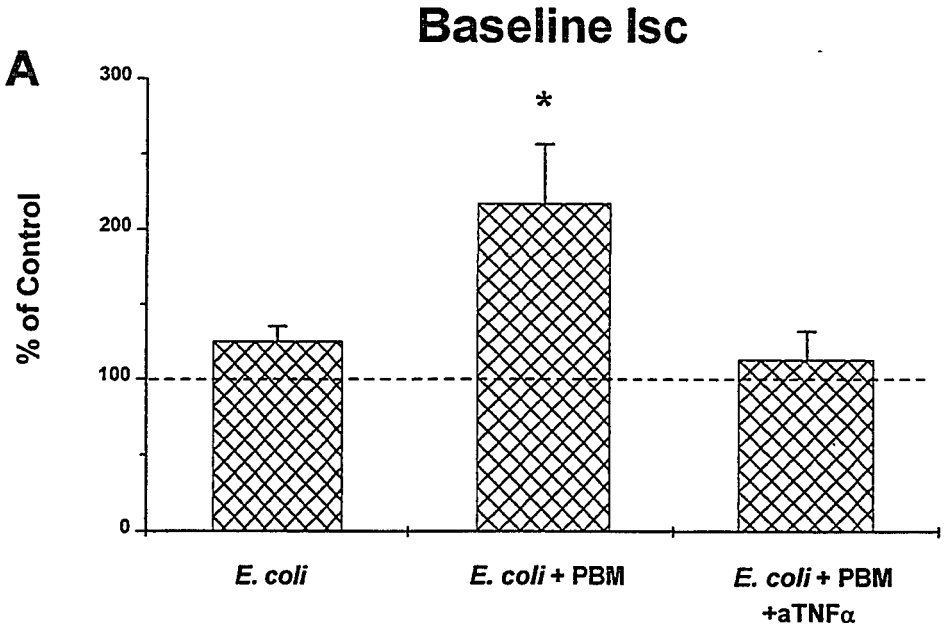

C
Resistance
% of Control
125
100
75
50
25
0
**
**
Normal
CD

Fig. 3.4. Percent changes from control values (T84 cells alone) in epithelial secretory responses; baseline Isc (A) and ΔIsc in response to forskolin (FSK) (B) of T84 monolayers after 48h of co-culture with control *E. coli* alone or *E. coli* and peripheral blood monocytes (PBM) in the presence or absence of anti-TNFα antibody (aTNFα) (n=6-8 donors). Values represent mean ± SEM with 2-4 monolayers per condition. **P*<0.05 compared to control (100%).


Baseline Isc
A
% of Control
300
200
100
0
*
E. coli
E. coli + PBM
E. coli + PBM
+aTNFα

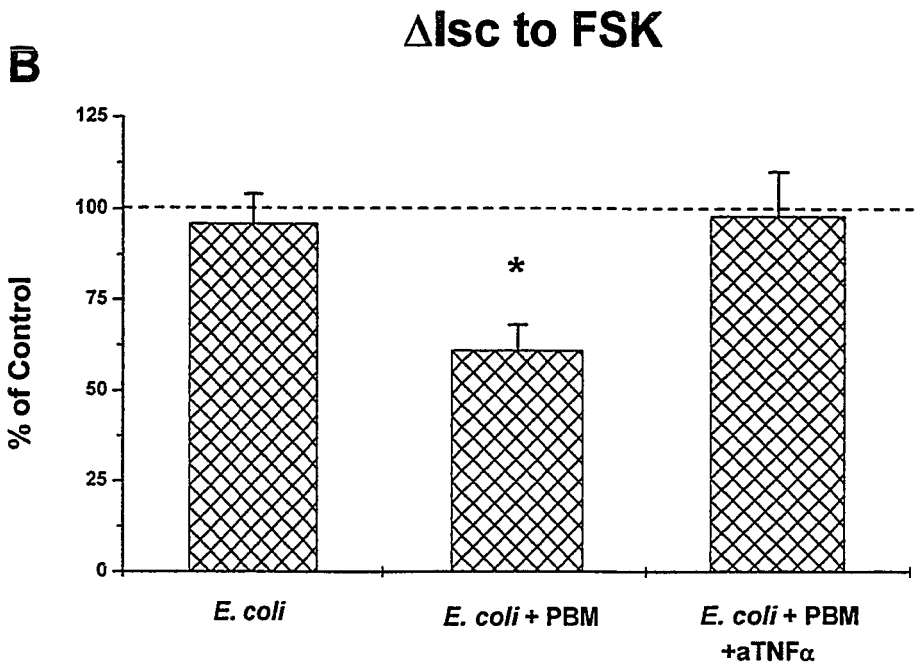
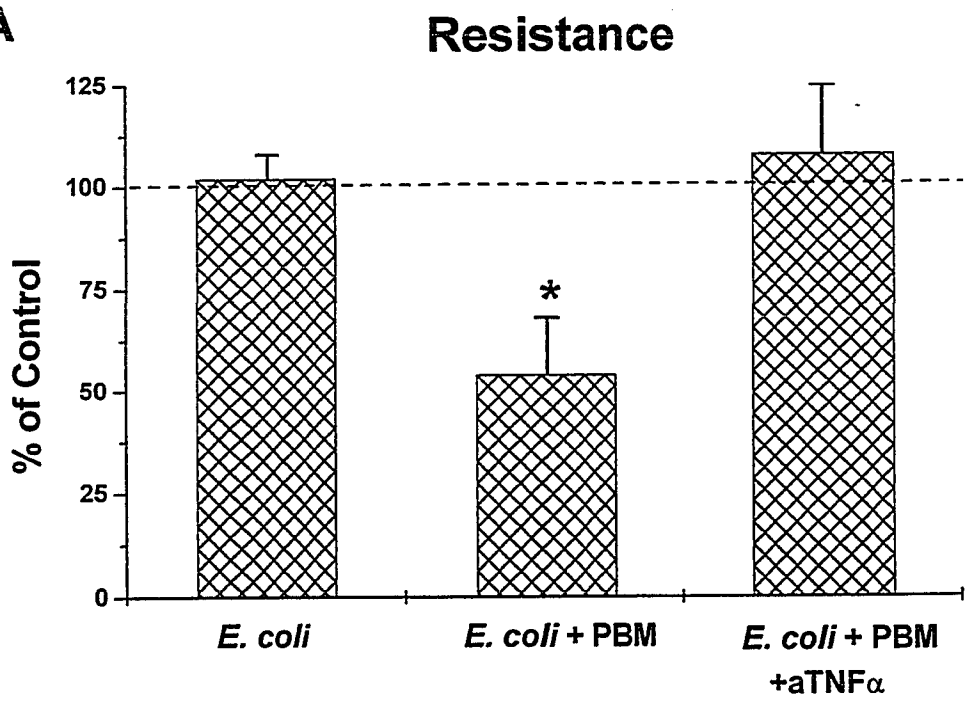
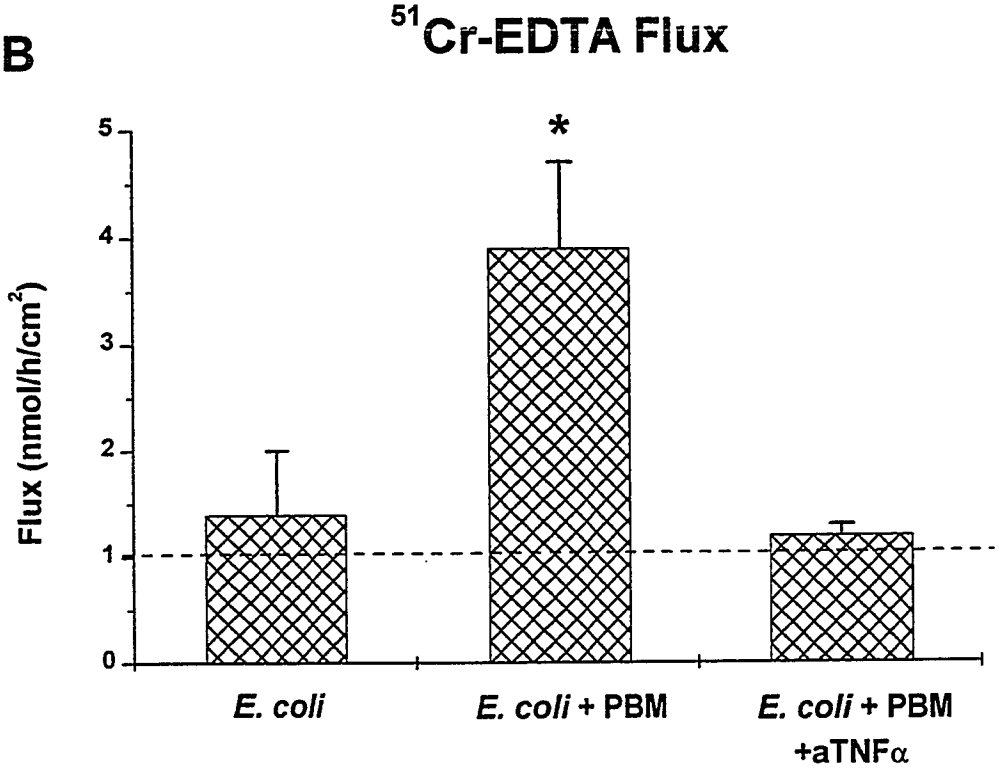

ΔIsc to FSK
B
% of Control
125
100
75
50
25
0
*
E. coli
E. coli + PBM
E. coli + PBM
+aTNFα

Fig. 3.5. Changes from control values (T84 cells alone) in epithelial barrier function measured by transepithelial resistance (A) and mucosal-to-serosal flux of ^{51}Cr-EDTA across T84 monolayers (B) after 48 h of co-culture with control *E. coli* alone or *E. coli* and peripheral blood monocytes (PBM) in the presence or absence of anti-TNFα antibody (aTNFα) (n=6-8 donors). Values represent mean ± SEM with 2-4 monolayers per condition. *P<0.05 compared to control (100%).

A


Resistance
% of Control
125
100
75
50
25
0
*
E. coli
E. coli + PBM
E. coli + PBM
+aTNFα

B


51Cr-EDTA Flux
Flux (nmol/h/cm2)
5
4
3
2
1
0
*
E. coli
E. coli + PBM
E. coli + PBM
+aTNFα

3.5 DISCUSSION

Inflammatory conditions of the gut are associated with increased heterogeneity and activation of intestinal macrophages, which represent a large fraction of the total macrophage pool of the body. Previous studies have shown that, unlike macrophages in normal mucosa[20], there is a sub-population of macrophages in the mucosa of patients with Crohn's disease which strongly express CD14[8], the receptor for bacterial LPS. Most of these cells also express the myelomonocytic marker L1, suggesting recent recruitment from peripheral blood[7]. Functional studies have shown that IBD macrophages seem to have an increased ability to undergo respiratory burst, present antigen and produce proinflammatory cytokines in response to LPS stimulation[7,21]. However, no data have been published on the direct effects of macrophages obtained from affected mucosa of patients with Crohn's disease on epithelial cell physiology. Moreover, no comparisons have been made between lamina propria mononuclear cells and peripheral blood monocytes isolated from normal subjects and patients with Crohn's disease, with regards to their ability to modulate intestinal epithelial function.

In the present study, we have demonstrated that PBM isolated from normal subjects and from patients with Crohn's disease were not spontaneously activated and, apart from a slight decrease in TER, did not alter epithelial cell physiology. LPS-induced activation of PBM, however, resulted in comparable physiological changes in T84 monolayers after co-culture with immune cells from both groups.

There was a significant increase in epithelial ion secretion, as demonstrated by elevation of baseline Isc. Activated PBM also increased epithelial permeability, as illustrated by a significant reduction in TER of the T84 monolayers. These findings demonstrate that normal PBM do not differ from those in Crohn's disease in their ability to alter epithelial cell physiology.

There was also no difference in the intrinsic capacity of normal PBM and Crohn's disease PBM to produce TNFα after stimulation with LPS. These data are in accordance with the findings of Bouma et al.[22] who reported similar TNFα production in blood cells derived from normal subjects and those with Crohn's disease.

We have documented here, for the first time, that intestinal macrophages from Crohn's disease differ from those of normal subjects in their ability to directly influence the intestinal epithelial physiology. LPMC isolated from normal intestinal mucosa did not induce either secretory (baseline Isc, ΔIsc in response to forskolin) or permeability changes (TER) in the T84 monolayers following a 48 h co-culture period. These cells did not respond directly to LPS nor did they induce epithelial physiological changes upon LPS exposure. In contrast, LPMC isolated from affected Crohn's mucosa were spontaneously activated and produced significant epithelial pathophysiology, as evaluated by a marked increase in the baseline Isc of the T84 monolayers and a significantly diminished response to forskolin after co-culture. The monolayers also displayed a substantially reduced TER, indicating an

impairment of their barrier property.

The fact that normal LPMC were incapable of activation by LPS could be explained by the lack of LPS receptor, CD14, on the surface of the macrophage population[8]. This limitation of the macrophage response is important in avoiding an inflammatory reaction to every day exposure of the epithelium to LPS from commensal bacteria in the normal intestine. In the inflamed intestine, however, the continuing influx of blood monocytes to the mucosa results in the presence of a sub-population of macrophages that is strongly CD14 positive and, consequently, sensitive to LPS. This sensitivity to LPS exposure becomes particularly important when associated with the damaged epithelial cells in active IBD or in the presence of a primary abnormality in epithelial tight junctions[23] when large quantities of LPS and other macromolecules may leak across the intestinal epithelium and gain access to the underlying mucosa. Exposure to LPS may induce the spontaneous activation of newly recruited macrophages in Crohn's disease mucosa, thus causing the pathophysiological changes (ion secretion and permeability) observed in the epithelial monolayers after co-culture.

Measurable TNFα was not detected in the supernatants of normal LPMC under either basal or LPS-stimulated conditions. In contrast, LPMC obtained from affected Crohn's disease mucosa spontaneously produced TNFα and were further stimulated by LPS to produce greater amounts of this cytokine. Infiltrating monocyte-like macrophages are believed to be an important source of TNFα in

Crohn's disease[9]. Depletion of macrophages from Crohn's disease LPMC results in a marked decrease in TNFα production, suggesting that intestinal macrophages are a major source of TNFα in the inflamed intestine[9].

TNFα is of particular importance in the pathophysiology of Crohn's disease. Several studies have shown increased TNFα protein and mRNA levels in biopsies obtained from patients with Crohn's disease[21]. Other studies have reported up-regulation of TNFα production in morphologically normal intestinal biopsies from patients with Crohn's disease[24], and adherence-separated macrophages from IBD mucosa stimulated by pokeweed antigen[25]. The increased level of TNFα may lead to enhanced activation of multiple mucosal cells, including macrophages, lymphocytes, and epithelial cells, and thereby contribute to mucosal damage in the intestine. In our experiments, a key role for TNFα in the pathogenesis of the epithelial abnormalities was also demonstrated. Both epithelial secretory and barrier defects were prevented when an antibody to TNFα was included in the co-culture. The importance of TNFα in the pathophysiology of Crohn's disease is further supported by recent clinical trials demonstrating the efficacy of anti-TNFα antibody in treatment of Crohn's disease[10-12].

There is considerable evidence from both animal models and clinical investigations supporting a pivotal role for luminal bacteria in the initiation or exacerbation of the chronic intestinal inflammation in Crohn's disease. Enteric bacteria, or their products, have been detected in inflamed mucosa of patients with

Crohn's disease[14]. Antibiotic treatment[26] or diversion of fecal stream[13] can reduce disease severity in some patients with active Crohn's disease. Finally, the spontaneous colitis that occurs in some murine transgenic and knockout models of colitis does not occur when the animals are housed under germfree conditions[15,27-29]. In contrast, little information is currently available on the role of commensal bacteria in altering epithelial function. This is partly due to the lack of a convenient experimental model to explore the normal flora-mucosal immune cell-epithelial cell interactions, in the regulation of intestinal epithelial physiology. Since LPMC from patients with Crohn's disease evoke epithelial abnormalities virtually identical those elicited by LPS-activated PBM, we utilized PBM to explore the effect of a non-pathogenic strain of *E. coli* (HB101) on epithelial physiology.

We have now demonstrated a direct role for a non-pathogenic strain of bacteria in the induction of intestinal epithelial pathophysiology via activation of monocytes/macrophages. We showed a marked increase in the baseline Isc of the T84 monolayers and a diminished response to forskolin after co-culture with luminal bacteria, but only in the presence of PBM. A substantially reduced TER, and enhanced permeability to ^{51}Cr-EDTA, also indicate impairment of the monolayer barrier property. These findings suggest that non-pathogenic commensal bacteria, in the presence of CD14+ macrophages below the epithelium, have the potential to alter intestinal epithelial secretory and barrier functions *via* immune activation. *In vivo*, similar events could result in a cascade of unregulated immune responses,

the consequences of which may be tissue damage leading to chronic inflammation. In this context, Duchmann et al.[5] have shown that mucosal mononuclear cells isolated from patients with IBD proliferate when exposed to autologous intestinal bacteria. They also demonstrated that animals previously tolerant to their own flora became intolerant after development of experimental colitis[1]. Taken together, these results support the concept that the normal tolerance towards autologous intestinal flora in healthy individuals is lost during inflammation associated with IBD.

In summary, monocyte/macrophages appear to be central to the induction of inflammation by normal bacteria and bacterial LPS, and can produce epithelial dysfunction. Unlike control LPMC, LPMC isolated from areas of active Crohn's disease are spontaneously activated and can induce epithelial pathophysiology. We have introduced a convenient tri-compartment co-culture model, using readily available peripheral blood monocytes to study the role of non-pathogenic enteric bacteria in the immune responses leading to epithelial dysfunction. This provides the researcher with a reproducible and manipulable simple model to assess the role of the enteric bacteria, mucosal macrophages and the epithelium, without facing the complexity of the mucosa or the need for surgical tissue.

We conclude that macrophages from affected bowel of patients with Crohn's disease are spontaneously altered compared to normal controls, and that their response to bacteria and bacterial products may lead to mucosal epithelial dysfunction through a mechanism which involves production of TNFα.

The authors wish to thank Ms Marysia Donnelly for providing blood from patients with Crohn's disease.

3.6 REFERENCES

1. Duchmann R, Schmitt E, Knolle P, Buschenfelde KMz, Neurath M: Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. Eur J Immunol 1996,26:934-8.

2. MacDonald TT: Breakdown of tolerance to the intestinal bacterial flora in inflammatory bowel disease. Clin Exp Immunol 1995,102:445-447.

3. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W: Interleukin-10 deficient mice develop chronic entrocolitis. Cell 1993,75:263-74.

4. Targan S, Deem RL, Liu M, Wang S, Nel A: Definition of a lamina propria T cell responsive state. Enhanced cytokine responsiveness of T cells stimulated through the CD2 pathway. J Immunol 1995,154:664-75.

5. Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K, Meyer Zum KH: Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol 1995,102:448-55.

6. Schreiber S, Heinig T, Thiele H, Raedler A: Immunoregulatory role of interleukin 10 in patients in inflammatory bowel disease. Gastroenterology 1995,108:1434-44.

7. Rugtveit J, Brandtzaeg P, Halstensen TS, Fausa O, Scott H: Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes. Gut 1994,35:669-74.

8. Grimm MC, Pavli P, Van de Pol E, Doe WF: Evidence for a $CD14^+$ population of monocytes in inflammatory bowel disease mucosa; implications for pathogenesis. Clin Exp Immunol 1995,100:291-7.

9. Reinecker HC, Steffen M, Witthoeft T, Pflueger I, Schreiber S, MacDermott RP, Raedler A: Enhanced secretion of tumor necrosis factor α, IL-6, and IL-1β by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clin Exp Immunol 1993,94:174-81.

10. Van Dullement HM, VanDeventer SJH, Hommes DW, Bijl HL, Jansen J, Tytgat GN, Woody J: Treatment of crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology

1995,109:129-35.

11. Targan SR, Hanauer SB, VanDeventer SJH, Mayer L, Present DH, Braakman TAJ, DeWoody KL, Schaible TF, Rutgeerts P: A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor α for crohn's disease. N Engl J Med 1997,337:1029-35.

12. Rutgeerts P, D'Haens G, Targan S, Vasiliauskas E, Hanauer SB, Present DH, Mayer L, Van Hogezand RA, Braakman TAJ, DeWoody KL: Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (Infliximab) to maintain remission in Crohn's disease. Gastroenterology 1999,117:761-9.

13. Rutgeerts P, Geboes K, Peeters M, Hiele M, Penninckx F, Aerts R, Kerremans R, Vantrappen G: Effect of fecal stream diversion on recurrence of Crohn's disease in the neoterminal illeum. Lancet 1991,338:771-4.

14. Klasen IS, Melief MJ, Van Halteren AGS: The presence of peptidoglycan-polysaccharide complexes in the bowel wall and the cellular responses to these complexes in Crohn's disease. Clin Immunol Immunopathol 1994,71:303-8.

15. Dianda L, Hanby AM, Wright NA, Sebesteny A, Hayday AC, Owen MJ: T cell receptor-αβ-deficient mice fail to develop colitis in the absence of a microbial environment. Am J Pathol 1997,150:91-7.

16. Zareie M, McKay DM, Kovarik GG, Perdue MH: Monocyte/macrophages evoke epithelial dysfunction: indirect role of tumor necrosis factor-α. Am J Physiol 1998,275(Cell Physiol. 44):C932-9.

17. Bull DM, Bookman MA: Isolation and functional characterization of human intestinal mucosal lymphoid cells. J Clin Invest 1977,59:966-74.

18. Philpott DJ, McKay DM, Sherman PM, Perdue MH: Infection of T84 cells with enteropathogenic *Escherchia coli* alters barrier and transport functions. Am J Physiol 1996,270 (gastrointest. Liver Physiol.): G634-45.

19. Bachman BJ: Derivation and genotype of some mutant derivatives of *Escherichia coli* K12. *Escerichia coli* and *Salmonella ryphimurium*. Edited by Neidhardt FC, Ingraham JL, Low KE, Magasanik B, Schacchter M, Umbarger HF. Washington DC, American Society for Microbiology, 1987, p. 1190-219.

20. Allison MC, Poulter LW: Changes in phenotypically distinct mucosal macrophage populations may be a prerequisite for the development of inflammatory bowel disease. Clin Exp Immunol 1991,85:504-9.

21. Cappello M, Keshav S, Prince C, Jewell DP, Gordon S: Detection of mRNAs for macrophage products in inflammatory bowel disease by in situ hybridization. Gut 1992,33:1214-9.

22. Bouma G, Oudkerk Pool M, Scharenberg JGM, Kolkman JJ, Von Blomberg BME, Scheper RJ, Meuwissen SGM, Pena AS: Differences in the intrinsic capacity of peripheral blood mononuclear cells to produce tumor necrosis factor alpha and beta in patients with inflammatory bowel disease and healthy controls. Scand J Gastroenterol 1995,30:1095-100.

23. Chadwick VS, Anderson RP: Inflammatory products of commensal bacteria and gastrointestinal disorders. Dig Dis 1990,8:253-68.

24. Reimund J, Wittersheim C, Dumont S, Muller CD, Kenney JS, Baumann R, Poindron P, Duclos B: Increased production of tumor necrosis factor-α, interleukin-1β, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. Gut 1996,39:684-9.

25. Rugtveit J, Nilsen EM, Bakka A, Carlsen H, Brandtzaeg P, Scott H: Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. Gastroenterology 1997,112:1493-505.

26. Sutherland LR, Singleton JW, Sessions J, Hanauer SB, Krawitt E, Rankin G, Summers R, Mekhjian H, Grenberger N, Kelly M: Double blind, placebo controlled trial of metronidazole in Crohn's disease. Gut 1991,32:1071-5.

27. Rath HC, Herfarth HH, Ikeda JS, Grenther WB, Hamm TE, Balish E, Taurog JD, Hammer RE, Wilson KH, Sartor RB: Normal luminal bacteria, specially *Bacteroides* species, mediate chronic colitis, gastritis and arthritis in HLA-B27/Human β_2 microglobulin transgenic rats. J Clin Invest 1996,98:945-53.

28. Contractor NV, Bassiri H, Reya T, Park AY, Baumgart DC, Wasik MA, Emerson SG, Carding SR: Lymphoid hyperplasia, autoimmunity, and compromised intestinal intraepithelial lymphocyte development in colitis-free gnobiotic IL-2 deficient mice. J Immunol 1998,160:385-94.

29. Sellon RK, Tonkonogy SL, Schultz M, Dieleman LA, Grenther WB, Balish E, Rennick D, Sartor RB: Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10 deficient mice. Infect Immun 1998,66:5224-31.

CHAPTER 4

IMPROVED EFFECTS OF NOVEL GLUCOCORTICOSTEROIDS ON IMMUNE-INDUCED EPITHELIAL PATHOPHYSIOLOGY

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Journal of Pharmacology and Experimental Therapeutics,

289: 1245-1249, 1999.

4.1 ABSTRACT

Glucocorticosteroids are a mainstay therapy in inflammatory bowel disease (IBD) and other chronic inflammatory conditions. However, severe systemic side effects are associated with their long term use. The new generation of glucocorticosteroids have a high degree of topical activity with reduced systemic effects due to rapid metabolism. We previously described an *in vitro* model of inflammation where monolayers of the human T84 colonic epithelial cell line displayed altered ion secretion and increased permeability after co-culture with endotoxin-activated monocytes (PBM). Here, we tested the effects of budesonide and two novel analogs, D5519 and S1316, on PBM-induced epithelial changes. Filter-grown T84 monolayers were co-cultured with activated PBM and single daily doses of drug were added to the luminal (physiological) side of the monolayer. Basal and stimulated epithelial ion transport (baseline short-circuit current, [Isc] and ΔIsc to forskolin, respectively) and barrier (transepithelial resistance) parameters were measured 48h later in Ussing chambers. D5519, S1316 and budesonide (10^{-9} to 10^{-7} M) dose-dependently inhibited the PBM-induced epithelial abnormalities, restoring normal resistance, decreasing the elevated baseline Isc and improving the reduced Isc response to forskolin. Of the drugs tested, D5519 was consistently the most potent and effective in inhibiting the PBM-induced epithelial irregularities. Coupled with a further improvement in their rate of hepatic inactivation, our findings indicate that the novel steroids, particularly D5519, will be

a valuable addition to current treatment strategies for IBD and other chronic inflammatory conditions.

4.2 INTRODUCTION

Glucocorticosteroids (GC) have been a major anti-inflammatory therapy for over four decades. However, despite their impressive immunosuppressive properties, the therapeutic value of GC is counterbalanced by a number of deleterious side effects including osteoporosis, adrenal insufficiency, hypertension and growth retardation in children. Attempts to overcome the systemic side-effects of steroid therapy, while maintaining therapeutic benefits, have led to the development of novel GC for topical treatment of inflammatory diseases. These new drugs are characterized by a high affinity for the GC receptor and an enhanced hepatic first-pass metabolism, resulting in products with an improved ratio between desirable high topical efficacy at the target and unwanted systemic steroid activity (Brattsand, 1990). The properties of these new GC make them particularly suitable for treating inflammation locally at mucosal surfaces, such as in the gastrointestinal tract and airways. In inflamed airways/lungs, the high affinity of these new GC for the GC receptor compensates for the great dilution of the drug over a large surface area, while in the inflamed intestine the enhanced hepatic first-pass inactivation is extremely important for reducing GC systemic side effects after leaving the target organ. As the prototype of these new GC, budesonide has proven to be beneficial in treating airway inflammation in patients with asthma and rhinitis (Brogden *et al.*, 1992; Pederson and O'Byrne, 1997). Budesonide has also been found to be effective in short term induction of remission during active Crohn's disease (Lofberg

et al., 1993; Greenberg *et al.,* 1994; Rutgeerts *et al.,* 1994; Campieri *et al.,* 1997). In an experimental study, we showed that budesonide could broadly inhibit T cell-mediated epithelial pathophysiology (McKay *et al.,* 1996a).

Despite its reported >90% first-pass metabolism, side effects such as adrenal insufficiency have been found to be associated with budesonide administration (Cui *et al.,* 1994). Recently, structural modifications to budesonide have led to newer GC which combine an even higher receptor affinity with a nearly complete first pass hepatic inactivation rate. The synthesis and basic pharmacological properties (affinity for the cytosolic GC receptor and inactivation rate) of one of these newer GC has been described in a recent paper by Thalén *et al.* (1998). This analog, D5519, together with another derivative, S1316, were shown to have twice the GC receptor affinity while being biotransformed 10-times more rapidly in human liver than budesonide, emphasizing a much lower systemic bioavailability after oral administration. However, while D5519 and S1316 display the desired physico-chemical characteristics, their usefulness in ameliorating immune-mediated disease symptoms (i.e. intestinal epithelial dysfunction) has not been examined.

We have recently described an *in vitro* model of inflammation, where co-culture of confluent monolayers of human T84 intestinal epithelial cells with endotoxin (or lipopolysaccharide [LPS]) activated monocytes (PBM), resulted in significant abnormalities in epithelial ion transport and barrier functions (Zareie *et*

al., 1998). These changes were largely abrogated by neutralization of TNFα. Utilizing this *in vitro* model, the present study was designed to compare the effects of D5519, S1316 and budesonide on immune-mediated changes in epithelial function. Our findings demonstrate beneficial properties of all three corticosteroids in this model system. However, D5519 was the most effective in reducing both TNFα production and immune-mediated epithelial pathophysiology.

4.3 MATERIALS AND METHODS

4.3.1 Cell Culture

Epithelial Cells

Human colonic epithelial cells (T84, passage 45-65) were seeded onto tissue culture-treated semi-permeable filter supports (0.4 μm pore size, 1.0 cm^2 surface area; Costar Corporation, Cambridge, MA) at 10^6 cells/filter and grown in culture media (equal volumes of Dulbecco's modified eagle medium (DMEM) and F12 medium, supplemented with 1.5% (v/v) HEPES, 2% (v/v) penicillin-streptomycin and 10% newborn calf serum) (all from Gibco Laboratories, Grand Island, NY). The culture media was changed daily. After culture for 7 days, confluent T84 monolayers consistently displayed transepithelial electrical resistances of greater than 1,000 Ω/cm^2 as measured by a volt meter (Millicel-ERS; Millipore Corporation, Bedford, MA). T84 monolayers displaying transepithelial electrical resistances of 1,000-2,000 Ω/cm^2 were used in these experiments.

Immune Cells

Human peripheral blood mononuclear cells (PBM) from healthy volunteers (male and female, ages 23-45) were isolated by one-step density centrifugation of whole blood over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and resuspended in fresh media at 10^6 cells/mL. The PBM population was obtained by

plastic plating of PBM (4h at 37°C) and subsequent removal of the non-adherent T and B cells. Fresh media was added to the adherent cells, which were then incubated for 18h at 37°C prior to use in co-culture studies. We have previously shown that >95% of the adherent immune cell population express CD14 (the LPS receptor) and have the appropriate size and granularity characteristics of PBM (Zareie *et al.*, 1998). Trypan blue exclusion revealed that >90% of PBM were viable following the co-culture period.

4.3.2 Immune Cell Activation

PBM were activated by *Salmonella minesota* LPS (10 ng/mL) (Sigma Chemical Co., St. Louis, MO) added to the culture media at the time of co-culture. Activation was assessed by the production of TNFα measured in culture media by ELISA (ELISA DuoSet, Genzyme Diagnostics, Cambridge, MA). Determinations were performed in duplicate using serial dilutions; the assay had a detection limit of 4 pg/mL.

4.3.3 Co-Culture Studies

Confluent T84 monolayers were co-cultured for 48h with PBM (~200,000 cells/well) placed in the basal compartment of the culture wells, as previously described (Zareie *et al.*, 1998). Control groups included 1) T84 monolayers alone, 2) T84 monolayers treated with GC, and 3) T84 monolayers co-cultured with non-

activated PBM.

4.3.4 Preparation of Corticosteroid Solutions

Budesonide, D5519 and S1316 were dissolved in absolute ethanol at a concentration of 10^{-2} M and stored as stock solutions at -20°C. Each drug was diluted to its required concentration (10^{-7} to 10^{-9} M) in fresh media on the day of use. Drugs were added to the apical compartment of the culture well in single daily doses during the 2-day co-culture period.

4.3.5 Ussing Chamber Experiments

Epithelial Ion Transport

Following co-culture, T84 monolayers were mounted in Ussing chambers as previously described (McKay *et al.,* 1996b). Epithelial monolayers were bathed in oxygenated Krebs buffer (37°C), containing 10 mM glucose as an energy source in the serosal buffer which was osmotically balanced by 10 mM mannitol in the mucosal buffer. The epithelial spontaneous potential difference (PD) was maintained at zero volts by the continuous injection of an external current by an automated voltage clamp (World Precision Instruments Inc, Sarasota, FL). This short-circuit current (Isc, in $\mu A/cm^2$) reflects net active ion transport across the preparation. Baseline Isc was recorded after a 15 min equilibration period. Stimulated ion secretion was measured by adding the adenylate cyclase-activating

agent, forskolin (10^{-5} M), or the cholinergic agonist, carbachol (10^{-4} M) (both from Sigma Chemical Co.), to the serosal side of the T84 monolayers and recording the maximum increase in Isc.

Epithelial Permeability

Electrical resistance is a measure of the barrier property of the epithelium to passive ion movement. Decreased resistance indicates an increase in permeability. At intervals during each experiment, PD across the monolayer was clamped at 1.0 mV (differential pulse method, 1 pulse/30 seconds), and the resulting change in current was used to calculate the transepithelial ion resistance (R, in Ω/cm^2) according to Ohm's law (Powell, 1981).

4.3.6 Cell Viability

T84 monolayer viability was assessed by measuring the release of lactate dehydrogenase (LDH) as described by Madara and Stafford (1989). After co-culture, T84 monolayers were removed and rinsed three times in fresh phosphate-buffered saline (PBS). Epithelial monolayers were lysed by immersing each filter in 0.1% (v/v) Triton-X 100 (Sigma Chemical Co.)/PBS for 30 min at room temperature followed by vigorous manual pipetting. The lysate was centrifuged at 500 rpm for 5 min and the supernatant was analyzed for LDH activity using an automated multiple point rate test (Kodak, Rochester, NY).

4.3.7 Statistics

Results are presented as mean ± SEM. Due to variability in absolute values between different batches of T84 cells, data were normalized to control values in each experiment (expressed as percentage of control). N values represent the number of experiments (different blood donors) in which 2-4 monolayers were examined for each condition. Data were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls comparison. Student's t-test was used for individual comparisons. Statistically significant differences were accepted at $p < 0.05$.

4.4 RESULTS

4.4.1 TNFα Production by PBM

Stimulation of PBM with 10 ng/mL LPS for 48h induced a significant increase in TNFα production (533 ± 68 pg/mL; n=8) compared to donor matched non-activated PBM (4 - 68 pg/mL). Activated PBM cultured in the presence of T84 cells receiving daily applications of D5519, S1316 and budesonide (10^{-7} M) displayed a significant reduction in TNFα production compared to activated PBM with no GC added (Fig. 4.1). At one log lower concentration (10^{-8} M), however, D5519 proved to be the most effective in reducing the TNFα production by 86 ± 2%, while budesonide was the least effective, reducing TNFα by 28 ± 5% (Fig. 4.1). At the lowest concentration (10^{-9} M), D5519 was the only GC to significantly inhibit the TNFα production.

4.4.2 PBM-induced Epithelial Pathophysiology

Ion Transport Abnormalities

T84 monolayers grown in the presence of non-activated PBM or GC alone displayed a baseline Isc of 0.8 ± 0.3 μA/cm^2; n=12 (range from 0.6 - 1.2 μA/cm^2), a value not significantly different from that of naïve monolayers. Therefore, naïve T84 monolayers were used as controls in further experiments. Co-culture with activated PBM for 48h evoked a significant increase in baseline Isc to 284-315%

of control values, indicating stimulated Cl^- secretion (Figs. 4.2A-C), as in our previous findings (Zareie *et al.,* 1998). Treatment of the monolayers with D5519, S1316 or budesonide dose-dependently inhibited this immune-mediated epithelial abnormality. Again, D5519 was the most potent GC compared to S1316 and budesonide under these conditions. T84 monolayers co-cultured with activated PBM plus D5519 at 10^{-8} M displayed completely normal baseline Isc values (Fig. 4.2A). At the same concentration, S1316 partially corrected the elevated baseline Isc (Fig. 4.2B), while budesonide showed partial diminution of the elevated baseline only at 10^{-7} M (Fig. 4.2C).

The ability of the epithelium to respond to forskolin was significantly reduced to ~70% of the control value: 63 ± 6 μA/cm^2; n=12 (range from 56-72 μA/cm^2) by co-culture of the monolayers with activated PBM. D5519 and S1316 ($\geq 10^{-8}$M) were equally effective in almost completely normalizing the reduced secretory response of the epithelium to forskolin to control values (Fig. 4.3A and 4.3B). In contrast, a beneficial effect of budesonide was observed only with a concentration of 10^{-7} M (Fig. 4.3C). Carbachol-induced ΔIsc was unaffected by co-culture of the monolayers with activated PBM (113±16 μA/cm^2 vs 97 ± 18 μA/cm^2 for controls).

Barrier Abnormalities

Control T84 monolayers displayed a transepithelial electrical resistance of 1557 ± 226 Ω/cm^2; n=12 (range from 1106-1784 Ω/cm^2). After 48h of co-culture

with activated PBM, the barrier function of T84 monolayers was significantly altered, as indicated by an ~ 40% reduction in transepithelial resistance of the monolayers compared to control monolayers. D5519 was the most effective GC tested, significantly inhibiting the activated PBM-induced reduction in epithelial resistance at concentrations $\geq 10^{-9}$ M (Fig.4.4A). In contrast, S1316 was effective only at 10^{-8} M (Fig. 4.4B). A 10-fold higher concentration of budesonide (10^{-7} M) was required to improve T84 monolayer resistance (Fig. 4.4C).

4.4.3 Epithelial Viability

After 48h, there was no significant difference in LDH released from T84 epithelial cells cultured in media alone or co-cultured with activated PBM (1497 ± 71 vs 1560 ± 88 U/L). Therefore, the effect of the GC was not investigated.

Fig. 4.1. Effect of GC (10^{-7} to 10^{-9} M) on TNFα production from LPS-activated monocytes after 48h of co-culture; (■) D5519; (□) S1316; (○) budesonide (n=2-4 donors for each GC with 4 replicates per donor and condition). Values represent mean ± SEM; *p<0.05 compared to LPS-activated monocytes (no drug) (100% = 533 ± 68 pg/mL); #p<0.05 compared to budesonide and S1316.

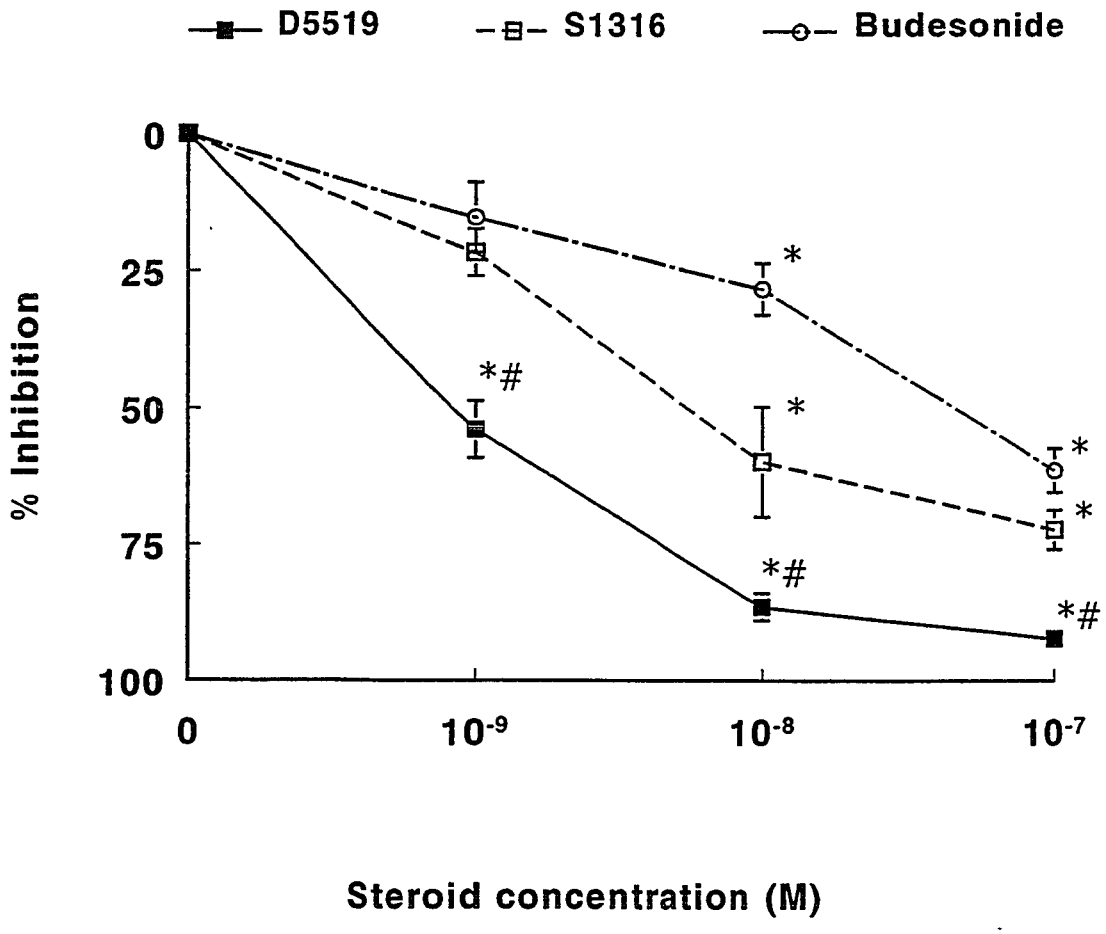

D5519
S1316
Budesonide
% Inhibition
0
25
50
75
100
0
10-9
10-8
10-7
*
*#
Steroid concentration (M)

Fig. 4.2. Percentage (%) change from control values (T84 cells alone) of epithelial baseline short-circuit current after 48h of co-culture with LPS-activated monocytes ± GC (10^{-7} to 10^{-9} M) added to the apical compartment of the culture well in single daily doses (n=5-6 experiments with 2-4 monolayers per experiment). Values represent mean ± SEM; # $p<0.05$ compared to no GC addition (0), *$p<0.05$ compared to control (100% = 0.8 ± 0.3 $\mu A/cm^2$).

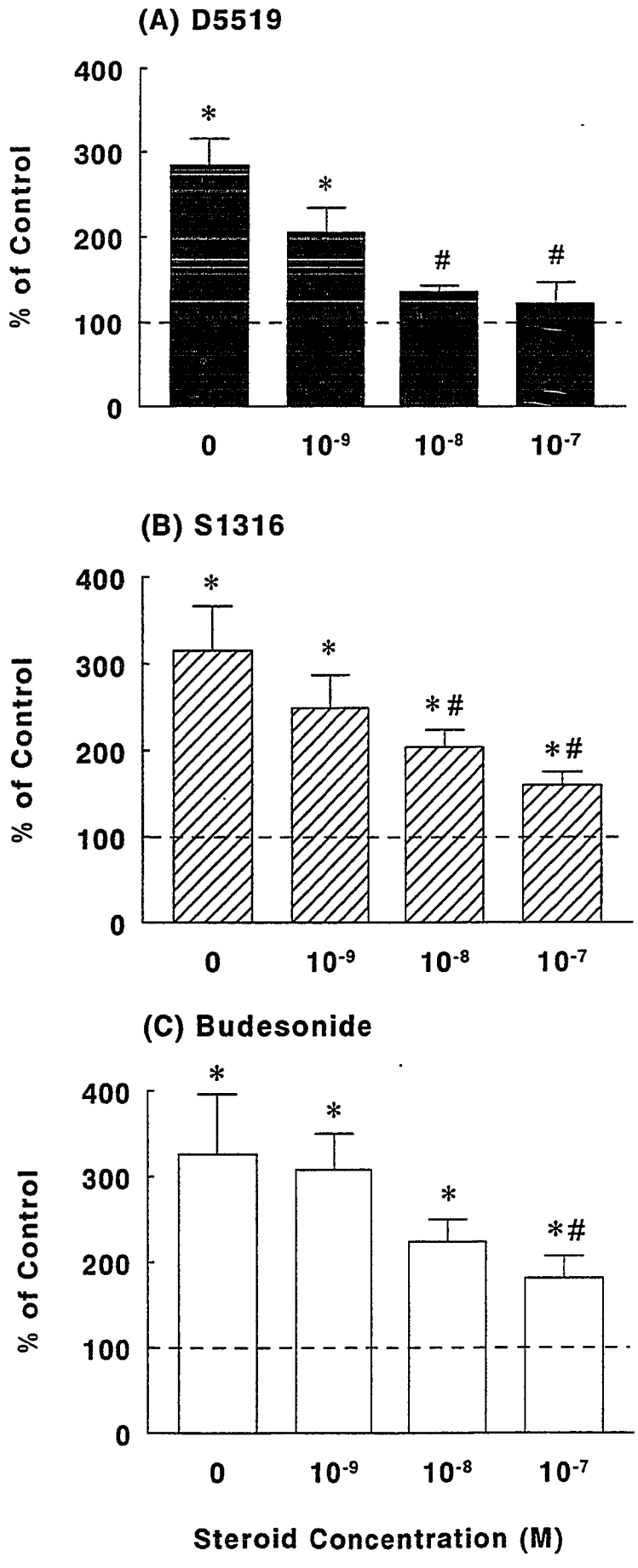

(A) D5519
400
300
200
100
0
% of Control
*
*
#
#
0
10-9
10-8
10-7
(B) S1316
400
300
200
100
0
% of Control
*
*
*#
*#
0
10-9
10-8
10-7
(C) Budesonide
400
300
200
100
0
% of Control
*
*
*
*#
0
10-9
10-8
10-7
Steroid Concentration (M)

Fig. 4.3. Percentage (%) change from control values (T84 cells alone) of epithelial secretory responses (ΔIsc) to forskolin (10^{-5} M) after 48h of co-culture with LPS-activated monocytes ± GC (10^{-7} to 10^{-9} M) added to the apical compartment of the culture well in single daily doses (n=5-6 experiments with 2-4 monolayers per experiment). Values represent mean ± SEM; # $p<0.05$ compared to no GC addition (0), *$p<0.05$ compared to control (100% = 63 ± 6 $\mu A/cm^2$).

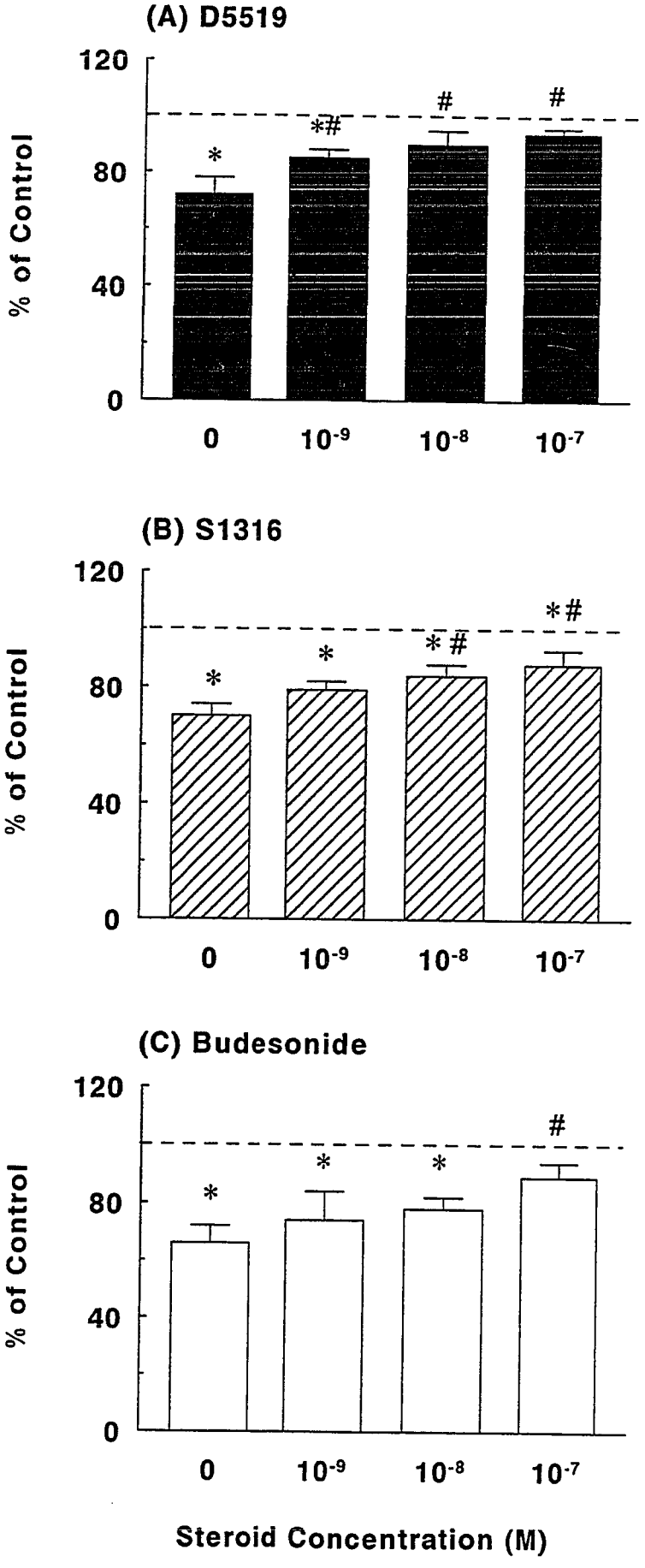
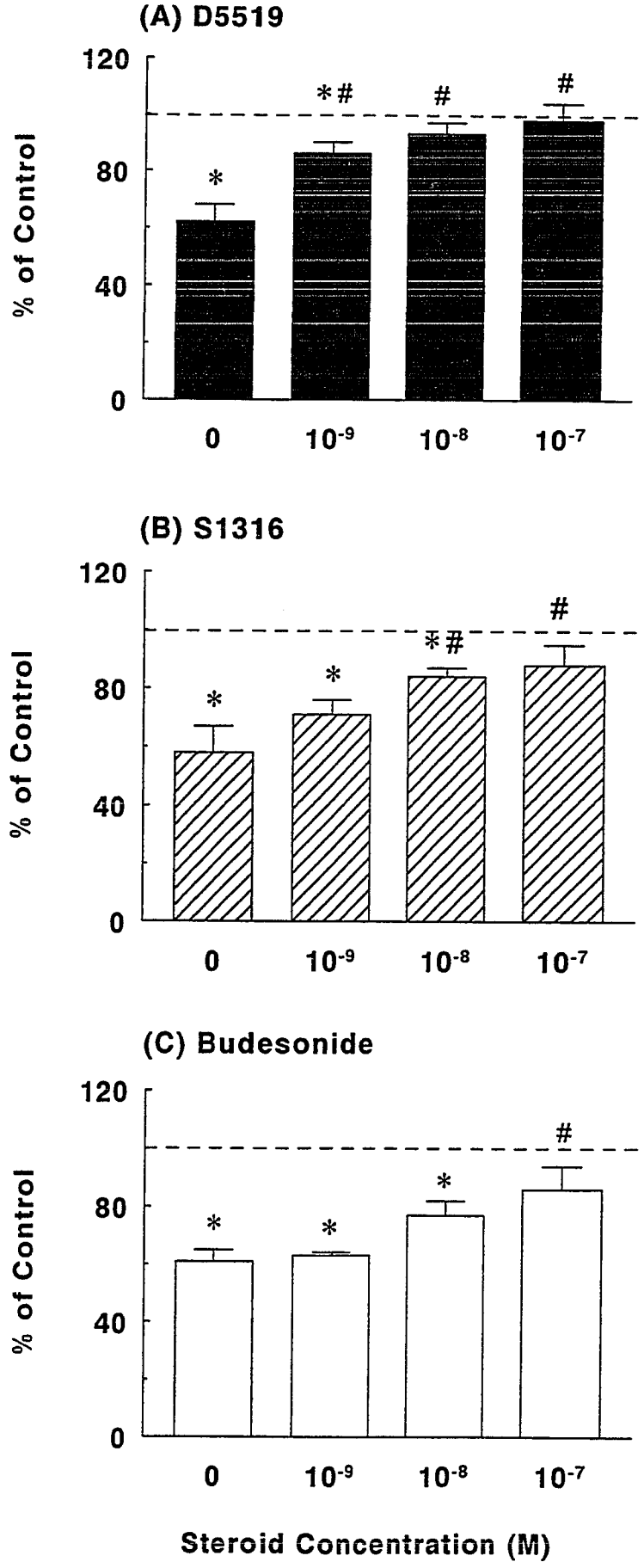

(A) D5519
120
80
40
0
% of Control
*
*#
#
#
0
10-9
10-8
10-7
(B) S1316
120
80
40
0
% of Control
*
*
*#
*#
0
10-9
10-8
10-7
(C) Budesonide
120
80
40
0
% of Control
*
*
*
#
0
10-9
10-8
10-7
Steroid Concentration (M)

Fig. 4.4. Percentage (%) change from control values (T84 cells alone) of transepithelial resistance after 48h of co-culture with LPS-activated monocytes ± GC (10^{-7} to 10^{-9}M) added to the apical compartment of the culture well in single daily doses (n=5-6 experiments with 2-4 monolayers per experiment). Values represent mean ± SEM; # $p<0.05$ compared to no GC addition (0), *$p<0.05$ compared to control (100% = 1557 ± 226 Ω/cm^2).


(A) D5519
120
80
40
0
% of Control
*#
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#
*
0
10-9
10-8
10-7
(B) S1316
120
80
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0
% of Control
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10-9
10-8
10-7
(C) Budesonide
120
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40
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10-9
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10-7
Steroid Concentration (M)

4.5 DISCUSSION

Inflammatory bowel disease (IBD) is characterized by altered epithelial physiology, typically increased permeability and diarrhea that may be due, at least in part, to altered regulation of ion secretion. Evidence from a number of studies has established that epithelial pathophysiology can be caused by activated immune cells (Madara *et al.,* 1993; Perdue and McKay, 1994; McKay *et al.,* 1996b). Among the immune cells, PBM play a central role in immune and inflammatory events in the intestinal mucosa. Recent studies have demonstrated that unlike in the intestine of normal individuals, resident macrophages in the intestinal lamina propria of patients with IBD express unusually high levels of CD14 (LPS receptor) on their cell surface, presumably due to rapid recruitment of monocytes from the circulation to the gut (Baldassano *et al.,* 1993; Rugtveit *et al.,* 1994; Grimm *et al.,* 1995). The newly recruited cells are more easily activated resulting in the production of excessive amounts of potent inflammatory mediators (Baldassano *et al.,* 1993; Rugtveit *et al.,* 1994). In accordance with these observations, we have recently described an *in vitro* model of inflammation in which co-culture of T84 human intestinal epithelial monolayers with activated PBM for 48h resulted in stimulated Cl^- secretion and impaired epithelial barrier function. In this model, PBM-derived TNFα was identified as a key factor in mediating these abnormalities (Zareie *et al.,* 1998). Here, we tested the effects of budesonide and two novel analogs, D5519 and S1316, on PBM-induced epithelial changes. Our data clearly demonstrate that

D5519 was the most effective GC in normalizing the PBM-induced epithelial ion transport and permeability irregularities, and this result is in accordance with its higher receptor affinity (Thalen *et al.,* 1998).

Budesonide has been used successfully in the treatment of asthma and allergic rhinitis (Pederson and O'Byrne, 1997). It has also been found to be effective in oral or rectal treatment of patients with IBD, most commonly Crohn's disease and ulcerative colitis, respectively (Campieri *et al.,* 1997; Greenberg *et al.,* 1994; Hanauer *et al.,* 1998). However, budesonide generally has similar efficacy to conventional steroids with high systemic availability (Hanauer *et al.,* 1998; Campieri *et al.,* 1997; Lofberg *et al.,* 1996; Rutgeerts *et al.,* 1994; Greenberg *et al.,* 1994) and it does not appear to markedly reduce the number of patients experiencing relapse after one year of treatment (Gross *et al.,* 1998; Greenberg *et al.,* 1996). Compared to other steroid regiments, fewer adverse effects, particularly less impact on the hypothalamic-pituitary-adrenal axis, have been reported in association with budesonide administration (Hanauer *et al.,* 1998; Campieri *et al.,* 1997). However, despite its high (>90%) first-pass metabolism in healthy people, as much as a 40% depression of plasma cortisol levels has been observed in Crohn's disease patients following administration of 9 mg/day of budesonide. This suggests that the extent of first pass metabolism is insufficient, especially in patients with active Crohn's disease who may have an impaired metabolism due to cytokine spillover from the intestine (Cui *et al.,* 1994; Greenberg *et al.,* 1994). The

need to produce potent GC that exhibit both greater receptor affinity (resulting in higher topical anti-inflammatory activity) and an enhanced hepatic inactivation rate (resulting in less systemic side effects) has led to the development of two new analogs of budesonide, D5519 and S1316. Utilizing our simplified model of intestinal inflammation, we have clearly shown for the first time in functional terms, that the new analogs of budesonide, particularly D5519, are more effective than budesonide itself in inhibiting the PBM-mediated epithelial abnormalities that are characteristic of intestinal inflammation. The steroid treatment protocol used in our co-culture system is compatible with that used in clinical settings, in that the steroids were added to the physiological (luminal) side of the epithelial monolayers.

Treatment of the T84 monolayers in the absence of PBM with budesonide, D5519 or S1316 did not alter epithelial ion secretion and did not affect the transepithelial resistance. This suggests that in our study, the inhibition of the immune-mediated epithelial pathophysiology was due to the effect of the drugs on PBM and not on the epithelial cells directly. In support of these observations, it has been reported that PBM activity, as measured by cytokine production, is steroid sensitive (Waage and Bakke, 1988; Linden and Brattsand, 1994; Oddera *et al.,* 1995). We have recently shown that PBM-derived TNFα is a critical mediator of epithelial pathophysiology in this *in vitro* model of intestinal inflammation (Zareie *et al.,* 1998). In the present study, the production of TNFα by PBM was significantly inhibited by steroid treatment, with D5519 being the most potent drug tested. The

potency of the 3 GC tested to inhibit TNFα production by PBM, correlated with their ability to prevent the epithelial dysfunction.

In summary, we have shown that PBM-induced epithelial abnormalities were inhibited in a dose-dependent manner by the addition into the co-culture of D5519, S1316 or budesonide. Our data also suggest that prevention of PBM-mediated epithelial pathophysiology was due to GC inhibition of PBM activation as shown by suppression of TNFα production. Finally, the novel analogs of budesonide, D5519 in particular, exhibited greater potency and efficacy in preventing epithelial dysfunction, when compared to budesonide. In conjunction with its higher GC receptor affinity and enhanced hepatic inactivation rate our results indicate a better therapeutic ratio especially for D5519, which should be of great advantage in topical therapy of inflammatory conditions of the intestine.

The authors thank P. Singh for expert technical assistance.

This research was funded in part by The Crohn's and Colitis Foundation of Canada, The Hospital for Sick Children Foundation, and Astra/Draco Pharma.

4.6 REFERENCES

1. Baldassano RN, Schreiber S, Johnston RB, Fu RD, Muraki T and MacDermott RP (1993) Crohn's disease monocytes are primed for accentuated release of toxic oxygen metabolites. *Gastroenterology* **105**: 60-66.

2. Brattsand R (1990) Overview of newer glucocorticosteroid preparations for inflammatory bowel disease. *Can J Gastroenterol* **4**: 407-414.

3. Brogden RN, McTavish D, Barnes PJ, Gross NJ, Juniper E and Laursen LC (1992) Budesonide: an updated review of its pharmacological properties, and therapeutic efficacy in asthma and rhinitis. *Drugs* **44**: 375-407.

4. Campieri M, Ferguson A, Doe W, Persson T, Nilsson L and The Global Budesonide Study Group (1997) Oral budesonide is as effective as oral prednisolone in active Crohn's disease. *Gut* **41**: 209-214.

5. Cui N, Friend DR and Fedorak RN (1994) A budesonide prodrug accelerates treatment of colitis in rats. *Gut* **35**: 1439-1446.

6. Greenberg GR, Feagan BG, Martin F, Sutherland LR, Thomson ABR, Williams CN, Nilsson L and Persson T (1994) Oral budesonide for active Crohn's disease. *N Engl J Med* **331**: 836-841.

7. Greenberg GR, Feagan BG, Martin F, Sutherland LR, Thomson ABR, Williams CN, Nilsson L, Persson T and The Canadian Inflammatory Bowel Disease Study Group (1996) Oral budesonide as maintenance treatment for Crohn's disease: a placebo-controlled, dose-ranging study. *Gastroenterology* **110**: 45-51.

8. Grimm MC, Pullman WE, Bennett GM, Sullivan PJ, Pavli P and Doe WF (1995) Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. *J Gastroentero Hepatol* **10**: 387-395.

9. Gross V, Andus T, Ecker KW, Raedler A, Loeschke K, Plauth M, Rasenack J, Weber A, Gierend M, Ewe K, Scholmerich J and The Budesonide Study Group (1998) Low dose oral pH modified release budesonide for maintenance of steroid induced remission in Crohn's disease. *Gut* **42**: 493-496, 1998.

10. Hanauer SB, Robinson M, Pruitt R, Lazenby AJ, Persson T, Nilsson L, Walton-Bowen K, Haskell LP and Levine JG (1998) Budesonide enema for the treatment of active, distal ulcerative colitis and proctitis: a dose ranging study. *Gastroenterology* **115**: 525-532.

11. Linden M and Brattsand R (1994) Effects of a corticosteroid, budesonide, on alveolar macrophage and blood monocyte secretion of cytokines: differential sensitivity of GM-CSF, IL-1β and IL-6. *Pulmonary Pharmacol* **7**: 43-47.

12. Lofberg R, Danielsson A and Salde L (1993) Oral budesonide in active Crohn's disease. *Aliment Pharmacol Ther* **7**: 611-616.

13. Lofberg R, Danielsson A, Suhr O, Nilsson A, Schioler R, Nyberg A, Hultcrantz R, Kollberg B, Gillberg R, Willen R and Persson T (1996) Oral budesonide versus prednisolone in patients with active extensive and left-sided ulcerative colitis. *Gastroenterology* **110**: 1713-1718.

14. Madara JL, Patapoff TW, Gillece-Castro B, Colgan SP, Parkos CA, Delp-Archer C and Mrsny RJ (1993) 5'-Adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. *J Clin Invest* **91**: 2320-2325.

15. Madara JL and Stafford J (1989) Interferon-gama directly affects barrier function of cultured intestinal epithelial monolayers. *J Clin Invest* **83**: 724-727.

16. McKay DM, Brattsand R, Wieslander E, Fung M, Croitoru K and Perdue MH (1996a) Budesonide inhibits T cell-initiated epithelial pathophysiology in an *in vitro* model of inflammation. *J Pharmacol Exp Therapeut* **277**: 403-410.

17. McKay DM, Croitoru K and Perdue MH (1996b) T cell-monocyte interactions regulate epithelial physiology in a coculture model of inflammation. *Am J Physiol* **270**: C418-C428.

18. Oddera S, Silvestri M, Sacco O, Lantero S, Morelli MC and Rossi G (1995) Evaluation of the inhibitory effects of budesonide on the mitogen-induced or the allergen-induced activation of blood mononuclear cells isolated from asthmatic patients. *Ann Allergy Asthma Immunol* **75**: 33-40.

19. Pederson S, and O'Byrne P (1997) A comparison of the efficacy and safety of inhaled corticosteroid in asthma. *Eur J Allergy Clin Immunol* **52**: 1-34.

20. Perdue MH and McKay DM (1994) Integrative immunophysiology in the intestinal mucosa. *Am J Physiol* **267**: G151-G165.

21. Powell DW (1981) Barrier function of epithelia. *Am J Physiol* **241**: G275-G288.

22. Rugtveit J, Brandtzaeg P, Halstensen TS, Fausa O and Scott H (1994) Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes. *Gut* **35**: 669-674.

23. Rutgeerts P, Loefberg R, Malchow H, Lamers C, Olaison G, Jewell D, Danielsson A, Goebell H, Ostergard Thompsen O, Lorenz-Meyer H, Hodgson H, Persson T and Seidegaard C (1994) A comparison of budesonide with prednisolone for active Crohn's disease. *N Engl J Med* **331**: 842-845, 1994.

24. Thalen BA, Halen BA, Axelsson BL, Andersson PH, Brattsand RL, Nylander B and Wickstorm L (1998) 6α-Fluoro- and 6α,9α- difluoro-11β,21-dihydroxy- 16α,17α- propylmethylene- dioxypregn- 4-ene-3,20-dione: synthesis and evaluation of activity and kinetics of their C-22 epimers. *Steroids* **63**: 37-43.

25. Waage A and Bakke O (1998) Glucocorticoids suppress the production of tumor necrosis factor by lipopolysaccharide-stimulated human monocytes. *Immunol* **63**: 299-302.

26. Zareie M, McKay DM, Kovarik GG and Perdue MH (1998) Monocyte/macrophages evoke epithelial dysfunction: indirect role of tumor necrosis factor-α. *Am J Physiol* **275**: C932-C939.

CHAPTER 5

DISCUSSION OF THE THESIS

DISCUSSION

Crohn's disease is a chronic inflammatory bowel disease that most commonly affects the terminal ileum and ascending colon. It is characterized by altered epithelial physiology, typically increased permeability and diarrhea that may be due, at least in part, to altered regulation of ion secretion. The precise nature of the initiating events involved in Crohn's disease remains unknown, although there is evidence from a variety of experimental models and clinical studies supporting the concept that inappropriate or exaggerated immune reactions are responsible for much of the pathogenesis of Crohn's disease (Duchmann *et al.,* 1996; Schreiber *et al.,* 1995; Kuhn *et al.,* 1993). The possibility that bacterial antigens or products of the normal intestinal flora may trigger inflammatory reactions is supported by a number of previous observations (Sellon *et al.,* 1998; Contractor *et al.,* 1998; Sartor, 1997; Dianda *et al.,* 1997; Rath *et al.,* 1996; MacDonald, 1995; Sadlack *et al.,* 1993; Kuhn *et al.,* 1993; Sutherland *et al.,* 1991; Rutgeerts *et al.,* 1991). However, no information is currently available on the direct role of commensal bacteria or their products in alterations of epithelial function in the context of immune cell activation. A number of studies have established that epithelial pathophysiology can be caused by activated immune cells such as lymphocytes and polymorphonuclear cells (McKay *et al.,* 1996; Perdue and McKay, 1994; Madara *et al.,* 1993). But few studies have considered the ability of cells of the monocyte/macrophage lineage to

directly affect epithelial function (Compeau *et al.*, 1994).

The purpose of the studies described in this thesis were: 1) to develop an *in vitro* co-culture model and explore monocyte-mediated changes in epithelial physiology; 2) to compare the cells of the monocyte/macrophage lineage from healthy volunteers *versus* those obtained from patients with Crohn's disease with respect to their ability to alter epithelial physiology upon activation by bacteria/bacterial products; 3) to examine if non-pathogenic control bacteria could induce changes in epithelial physiology in the presence of monocytes; and 4) to investigate the efficacy of recently developed therapeutic agents (anti-TNFα and novel glucocorticosteroids) to inhibit of the monocyte-mediated epithelial pathophysiology.

5.1 PBM EVOKE EPITHELIAL DYSFUNCTION UPON ACTIVATION BY BACTERIAL PRODUCTS

In the first part of this project, we used an *in vitro* co-culture model system to demonstrate that monocytes activated with common bacterial products, in the absence of other immune cell types, can alter epithelial ion transport and permeability.

Bacterial LPS is present in large quantities in the intestinal lumen. When exposed to LPS, monocytes synthesize an array of proinflammatory mediators

(Surette *et al.,* 1993; Dentener *et al.,* 1993; Lord *et al.,* 1991; Nathan, 1987) that are capable of inducing local tissue damage through their interactions with T cells, leukocytes and endothelial cells (Perdue and McKay, 1996). The bacterial tripeptide, fMLP (Sherriff *et al.,* 1992), induces monocyte chemotaxis and adherence as well as the production of oxygen radicals and pro-inflammatory eicosanoids (Nast and LeDuc, 1988). Since LPS and fMLP are usually present simultaneously in the gut lumen, we chose to add both agents directly to monocytes (mimicking events following their uptake from the lumen) and then determine the consequence of these agents on monocyte activation and epithelial ion transport and permeability.

Our study clearly showed that as few as 5 x 10^4 monocytes activated by LPS/fMLP can evoke a luminally directed active Cl^- secretion (as shown by the elevation of T84 baseline Isc) and significantly increase epithelial ionic permeability (as illustrated by a significant reduction in transepithelial resistance of the monolayer and increased transepithelial flux of the inert probe, ^{51}Cr-EDTA) after 48h. This co-culture model was the first study to show increased epithelial baseline secretion in response to monocyte activation. These findings suggest that cells of monocyte/macrophage lineage are potent cells which may mediate some of the pathophysiology in inflammatory disorders of the gut; and can be added to the growing list of immune cells (T cells, mast cells, neutrophils and eosinophils) (McKay *et al.,* 1996; Perdue and McKay, 1994; Madara *et al.,* 1993; Barrett, 1991)

that regulate epithelial physiology. Complementing our findings, it has been shown that LPS-stimulated alveolar macrophages alter ion transport in isolated rat lung epithelial cells (Compeau *et al.,* 1994).

5.2 COMPARATIVE ANALYSIS OF NORMAL PBM AND LPMC WITH PBM AND LPMC FROM CROHN'S DISEASE

Resident intestinal macrophages are located close to the basal membrane of the intestinal epithelium and represent the first line of defense by immune cells. Additional monocytes may be attracted to the intestinal mucosa during inflammation by locally produced MCP-1 (Reinecker *et al.,* 1995). Recent studies have demonstrated the appearance of macrophage sub-populations with a different phenotype in IBD mucosa (Rugtveit *et al.,* 1994; Mahida *et al.,* 1989) which unlike resident macrophages in normal intestine, express high levels of the LPS receptor, CD14 (Grimm *et al.,* 1995a). Functional studies have shown that these newly recruited cells seem to have increased ability to undergo respiratory burst, enhanced antigen presenting ability and to have a highly increased potential to produce proinflammatory cytokines in response to LPS stimulation (Rugtveit *et al.,* 1994; Reinecker *et al.,* 1993; Cappello *et al.,* 1992). However, to our knowledge, no data have been published on the direct effects of such cells obtained from affected mucosa of patients with Crohn's disease on epithelial physiology, and no

comparisons have been made between the LPMC and the PBM isolated from normal volunteers and patients with Crohn's disease, in regard to their ability to influence intestinal epithelial function.

In our initial study we demonstrated that PBM activated by common bacterial products can stimulate Cl^- secretion, alter ion transport responses and impair the barrier function of the epithelium. Increased ion secretion and abnormal epithelial permeability are hallmarks of the epithelial pathophysiology in Crohn's disease. Therefore, to examine if monocyte/macrophages mediate some of the epithelial pathophysiology observed in Crohn's disease, we utilized the same *in vitro* co-culture model to determine: 1) if there is a difference in the ability of PBM (± LPS) obtained from patients with Crohn's disease to alter epithelial physiology vs PBM obtained from normal humans and 2) to compare the effect on epithelial physiology of LPMC from control non-inflamed intestine *versus* LPMC isolated from intestine of patients with Crohn's disease.

We demonstrated that PBM isolated from patients with Crohn's disease were not spontaneously activated and did not alter epithelial physiology. LPS activation of Crohn's PBM, however, resulted in physiological changes in T84 monolayers after the co-culture (significant increase in epithelial ion secretion demonstrated by elevation of T84 baseline Isc, and increased epithelial permeability to ions illustrated by a significant reduction in TER). These epithelial changes were similar to those induced following co-culture with PBM from normal volunteers. Our

findings clearly illustrated that normal PBM are not different from Crohn's PBM in their ability to affect epithelial physiology.

In our second study, we clearly demonstrated for the first time that colonic LPMC from patients with Crohn's disease differ from normal colonic LPMC in their ability to directly influence intestinal epithelial physiology. We showed that the LPMC isolated from normal intestinal mucosa did not induce any secretory (baseline Isc, ΔIsc in response to forskolin) or permeability changes (TER) in the T84 epithelium following the 48 h co-culture period. In addition, LPS did not have any stimulating effects on these cells and the epithelial physiological responses remained unchanged upon LPS exposure. LPMC isolated from actively inflamed mucosa of patients with Crohn's disease, on the other hand, were spontaneously activated and caused significant epithelial pathophysiology. There was a marked increase in the baseline Isc of the T84 monolayers accompanied by a significantly diminished response to forskolin after co-culture with LPMC from Crohn's disease mucosa. The monolayers also displayed a substantially reduced TER, indicating impairment of their barrier properties. Addition of LPS had no significant effects on already altered epithelial physiology.

The fact that normal LPMC were incapable of activation by LPS could be explained by the lack of LPS receptor, CD14, on the surface of the intestinal resident macrophage population (Grimm *et al.,* 1995a). This limitation of the macrophage response is important in avoiding an inflammatory reaction due to

every day exposure of the epithelium to LPS of commensal bacteria in the normal intestine. In the inflamed intestine, however, a second population of macrophages exists that is strongly CD14 positive and consequently sensitive to LPS. An impairment of the epithelial barrier due to epithelial cell damage in active IBD could result in increased uptake of LPS and other macromolecules across the intestinal epithelium. This coupled with the increased sensitivity of the mucosal macrophages to LPS exposure may lead to synthesis and release of potent proinflammatory cytokines such as TNFα, tissue damaging enzymes, lipid inflammatory mediators and reactive oxygen metabolites from macrophages, all of which have been shown to be produced in excessive amounts in IBD tissue (Rugtveit *et al.,* 1995; Reinecker *et al.,* 1993; Cappello *et al.,* 1992; Mahida *et al.,* 1989). Therefore, the results of our second study suggest that ongoing exposure to LPS may induce spontaneous activation of the newly recruited, $CD14^+$ macrophages in Crohn's disease mucosa, thus causing the pathophysiological changes (ion secretion and permeability) in the epithelial monolayers after the co-culture. Modeling this scenario *in vitro*, we clearly demonstrated that the spontaneous activation of colonic macrophages from Crohn's disease, possibly by bacteria/bacterial products, has significant consequences for epithelial ion transport and permeability functions. These cells certainly have the ability to directly influence intestinal epithelial physiology in a manner similar to activated circulating monocytes and mediate some of the pathophysiology observed in Crohn's disease.

5.3 A NOVEL *IN VITRO* MODEL OF INFECTION/INFLAMMATION:

Evidence for the Direct Role of Non-pathogenic Control Bacteria in Induction of Epithelial Pathophysiology *via* Immune Cell Activation

Evidence from a number of studies suggests that normal luminal bacteria may be involved in the initiation and exacerbation of chronic intestinal inflammation in Crohn's disease. Clinical experience has shown that antibiotic treatment (Peppercorn, 1993; Sutherland *et al.,* 1991; Jakobovits and Schuster, 1984) or diversion of fecal stream (Rutgeerts *et al.,* 1991) reduces disease activity in Crohn's disease patients. Levels of antibodies against intestinal bacteria are elevated in the serum of individuals with IBD (Sartor, 1997) and enteric bacteria or their products have been detected within the inflamed mucosa in Crohn's disease (Klasen *et al.,* 1994). In murine models of spontaneous colitis including IL-2 and IL-10 knock out mice, or the HLA-B27 transgenic rats, colitis does not occur if the animals are maintained under germ free conditions (Sellon *et al.,* 1998; Contractor *et al.,* 1998; Dianda *et al.,* 1997; Rath *et al.,* 1996; Sadlack *et al.,* 1993; Kuhn *et al.,* 1993). In addition, Duchmann et al. have reported that mucosal mononuclear cells isolated from patients with IBD proliferate when exposed to autologous intestinal bacteria (Duchmann *et al.,* 1995). Despite the considerable evidence on the association of commensal flora and gut inflammation in Crohn's disease, no experimental model has been described to explore the interactions of normal flora with mucosal

immune cells and epithelial cells.

In the second part of this project, we demonstrated striking similarities between epithelial pathophysiology induced by LPMC from patients with Crohn's disease (in the absence of LPS) and that induced by LPS-activated PBM. Thus, we utilized circulating PBM to explore the effects on epithelial physiology of a non-pathogenic control strain of *E. coli* (HB101), added to the physiological (luminal) side of the T84 monolayers.

This study is the first to provide evidence for a direct role of non-pathogenic bacteria in the induction of intestinal epithelial pathophysiology via monocyte/macrophage activation. We showed that non-pathogenic control bacteria, in the presence of $CD14^+$ monocyte/macrophages, have the potential to alter the secretory and barrier functions of a model intestinal epithelium *via* initiating immune activation. *In vivo*, similar events could result in a cascade of unregulated immune responses, the consequences of which may be tissue damage leading to chronic inflammatory disease. Our results provide additional information on how, in a susceptible individual, an immune response to a control bacteria which does not normally cause epithelial dysfunction, could significantly alter the physiology of the epithelium.

5.4 EXAMINATION OF THE EFFECTS OF NOVEL THERAPEUTIC AGENTS IN *IN VITRO* CO-CULTURE MODEL OF INTESTINAL INFLAMMATION

The *in vitro* co-culture system described in this thesis provides a useful model not only to study the immune-mediated epithelial pathophysiology, but also allows to examine the effects of novel therapeutics on epithelial dysfunction. Two potential treatments for inflammatory diseases of the gut are discussed below.

5.4.1 Anti-TNFα Therapy: Role of TNFα in the Monocyte/macrophage Mediated Epithelial Pathophysiology

Stimulation of PBM by bacteria/bacterial products leads to the production of an array of proinflammatory cytokines (Surette *et al.,* 1993; Dentener *et al.,* 1993; Lord *et al.,* 1991; Nathan, 1987), and among these, TNFα is of particular importance in the pathophysiology of Crohn's disease. Several studies have reported increased TNFα protein and mRNA levels in biopsies from IBD patients, particularly in Crohn's disease (Plevy *et al.,* 1997; MacDonald *et al.,* 1990). Significantly increased concentrations of TNFα have been reported in stools of children with active chronic IBD (Braegger *et al.,* 1992). Other studies have also reported a rise in circulating TNFα and the soluble TNFα receptor (p55) in patients with active IBD which were significantly correlated with the clinical and/or laboratory

measures of disease activity (Gardiner *et al.,* 1995; MacDonald *et al.,* 1990).

In exploring the role of TNFα in our model of epithelial dysfunction, we demonstrated significant production of TNFα by non-activated LPMC obtained from mucosa of patients with Crohn's disease, as well as circulating PBM activated by bacteria or bacterial products. Neutralization of TNFα in the co-culture model by addition of an anti-TNFα antibody reduced the T84 baseline Isc response to control levels and significantly improved the T84 transepithelial resistance. In addition, anti-TNFα treatment inhibited the increased epithelial permeability to ^{51}Cr-EDTA evoked by culture with activated PBM. The correction of the abnormal epithelial function by anti-TNFα suggested that TNFα directly affected T84 physiology, alone or in concert with other immune mediators. In our initial study, we showed that addition of recombinant TNFα at the concentrations measured in the supernatant from activated PBM did not affect epithelial physiology, while cell-free conditioned medium from activated PBM (and spontaneously activated LPMC from Crohn's disease in our second study) were equally effective in producing the epithelial abnormalities after co-culture. Moreover, neutralization of TNFα inhibited the epithelial abnormalities only when anti-TNFα was added in the presence of PBM but not when added to PBM conditioned medium. This suggested that the TNFα was affecting the PBM in an autocrine manner, and not the epithelium directly. Our findings are in agreement with a number of studies which have not been able to demonstrate a direct action of TNFα on T84 function following an acute exposure

(≤72h; 3-6 ng/ml) (Taylor *et al.,* 1997; McKay *et al.,* 1996; Madara and Stafford, 1989). However, there are some studies which suggest a direct role for TNFα in regulating the epithelial physiology, but only in the presence of other mediators (IFNγ) (Taylor *et al.,* 1998; Madsen *et al.,* 1997) or in epithelial cells other than T84 (Rodriguez *et al.,* 1995).

The observation that recombinant TNFα added to non-activated PBM resulted in elevated baseline Isc and decreased transepithelial resistance of T84 monolayers, and the fact that these results were very similar to those evoked by LPS-activation of PBM, provided further support for the hypothesis that TNFα affected PBM in an autocrine manner, causing the release of other as yet unidentified PBM-derived mediator(s), the net result of which was altered epithelial function. Integrating our findings with previous studies, we demonstrated a key role for TNFα in the pathogenesis of the epithelial abnormalities. The increased production of TNFα by monocyte/macrophages may modulate epithelial function indirectly perhaps *via* enhanced activation of many cell types present in the intestinal mucosa, including macrophages, lymphocytes, and epithelial cells, and may contribute to the mucosal epithelial damage within the intestine. We also showed that epithelial abnormalities could be successfully abrogated by inclusion of a neutralizing anti-body to TNFα. The therapeutic value of TNFα neutralization is supported by a recent multi-center, placebo-controlled trials, demonstrating that anti-TNFα antibody (cA2 antibody; same antibody used in this study) treatment

resulted in prolonged clinical improvement in some patients with Crohn's disease (Rutgeerts *et al.,* 1999; Targan *et al.,* 1997; Van Dullement *et al.,* 1995).

5.4.2 Inhibition of the Monocyte/macrophage Mediated Epithelial Pathophysiology by Novel Glucocorticosteroids

Glucocorticosteroids are of considerable benefit in the treatment of severe cases of IBD. However, serious side effects are associated with their long term use. Recently, novel glucocorticosteroids have been developed that have a high affinity for the glucocorticoid receptor and an enhanced hepatic first-pass metabolism and thus, have an improved ratio between a high topical efficacy and unwanted systemic toxicity (Brattsand, 1990). Two such compounds are the novel analogs of budesonide, D5519 and S1316. Budesonide has been beneficial in treating airway inflammation (Pederson and O'Byrne, 1997). It has also been effective in short term induction of remission during active Crohn's disease (Campieri *et al.,* 1997; Rutgeerts *et al.,* 1994; Greenberg *et al.,* 1994; Lofberg *et al.,* 1993). However, budesonide had efficacy similar to other conventional steroids (Campieri *et al.,* 1997; Lofberg *et al.,* 1996; Rutgeerts *et al.,* 1994; Greenberg *et al.,* 1994) and despite its high (>90%) first-pass metabolism in healthy individuals, as much as 40% depression of plasma cortisol levels has occurred in patients with Crohn's disease (Cui *et al.,* 1994). The two analogs of budesonide, D5519 and S1316, have a greater receptor affinity (resulting in higher topical anti-inflammatory

activity) and a nearly complete first-pass hepatic inactivation rate (resulting in less systemic side effects) (Thalen *et al.,* 1998). In the third part of this project, we utilized our co-culture model of intestinal inflammation to test the usefulness of these novel steroids in preventing the PBM-mediated epithelial pathophysiology and to compare the effectiveness of these compounds to budesonide. We clearly showed (for the first time in functional terms) that these new analogs of budesonide, particularly D5519, are more effective than budesonide itself in inhibiting the PBM-induced epithelial abnormalities (altered ion secretion and impaired barrier function) that are characteristics of intestinal inflammation. Our data also suggested that the beneficial effects of these steroids on epithelial pathophysiology were due to inhibition of PBM activation. The production of TNFα by PBM was significantly inhibited by steroid treatment. The potency of the three corticosteroids tested to inhibit TNFα production correlated with their ability to prevent the epithelial dysfunction. Other studies have also reported that PBM activation, as measured by cytokine production, is steroid sensitive (Oddera *et al.,* 1995; Linden and Brattsand, 1994; Waage and Bakke, 1988). In conjunction with their greater receptor affinity and enhanced hepatic inactivation rate, our results indicated a better therapeutic ratio, especially for D5519. This could be of great advantage in topical treatment of inflammatory diseases of the gut such as Crohn's disease.

In addition, the steroid treatment protocol used in our study is comparable to that used in clinical setting, in that the steroids were added to the physiological

(luminal) side of the epithelial monolayers. Thus, the co-culture model of intestinal inflammation described in this thesis provides an excellent tool to test for the efficacy of novel drugs being developed for treatment of IBD.

5.5 SIGNIFICANCE OF THE RESEARCH PRESENTED IN THIS THESIS

Despite extensive research in different areas, the precise nature of the factors which initiate or induce relapse in Crohn's disease remains unknown. Among a variety of factors implicated in the pathogenesis of Crohn's disease, it has been suggested that much of the pathophysiology and epithelial damage is due to an inappropriate or exaggerated immune reaction (Duchmann *et al.,* 1996; Schreiber *et al.,* 1995; MacDonald, 1995; Kuhn *et al.,* 1993). Indeed, a number of studies have established that epithelial dysfunction can be caused by immune cells such as lymphocytes and polymorphonuclear cells (McKay *et al.,* 1996; Perdue and McKay, 1994; Madara *et al.,* 1993). There is also evidence suggesting that normal intestinal flora or bacterial products may trigger or contribute to inflammatory reactions leading to IBD (Sellon *et al.,* 1998; Contractor *et al.,* 1998; Sartor, 1997; Dianda *et al.,* 1997; Rath *et al.,* 1996; Sadlack *et al.,* 1993; Kuhn *et al.,* 1993; Sutherland *et al.,* 1991; Rutgeerts *et al.,* 1991). However, in studies of chronic intestinal inflammation in Crohn's disease, three fundamental questions still remain to be answered: 1) the nature of the antigens originally initiating the inappropriate immune response; 2) the immune cells primarily contributing to the inflammatory process and 3) the consequences of immune activation on intestinal epithelial physiology.

The research presented in this thesis has provided some insights to the answers to the above questions. My research has been focused on cells of the

monocyte/macrophage lineage and their role in epithelial pathophysiology similar to that seen in Crohn's disease. Several studies have shown that macrophages in the lamina propria of the intestine of patients with Crohn's disease are different from macrophages in the normal intestine in their phenotype and intrinsic capacity to produce and secrete a number of inflammatory mediators. My research, however, is the first to define a critical role for monocyte/macrophages in the regulation of intestinal epithelial physiology. The highlights of the studies included in this thesis are as follows:

1. We have provided sufficient evidence to conclude that PBM, when activated by common bacterial products, are potent cells in inducing physiological abnormalities (altered ion secretion and impaired barrier function) in a model intestinal epithelium.
2. There is no difference in the intrinsic capacity of PBM from individuals with Crohn's disease and those from healthy individuals to cause epithelial pathophysiology, upon activation by common bacterial products.
3. LPMC obtained from non-inflamed (carcinoma) tissue do not affect the epithelial physiological responses, even upon LPS exposure.
4. Unlike normal LPMC, LPMC from patients with Crohn's disease are spontaneously activated and have the ability to directly influence epithelial pathophysiology, involving significant abnormalities in the secretory and barrier function of the epithelium.

5. The epithelial pathophysiology induced by non-activated LPMC from Crohn's disease is strikingly similar to the pathophysiology induced by LPS-activated PBM. Thus, blood provides an easily accessible source of CD14+ monocytes, similar to the newly recruited $CD14^+$ macrophages in the inflamed mucosa of patients with Crohn's disease.

6. We have described for the first time an *in vitro* co-culture model, utilizing normal circulating PBM to explore the effect of non-pathogenic control bacteria (do not cause epithelial pathophysiology under normal conditions) on epithelial physiology in the context of immune cell activation.

7. We have demonstrated, for the first time, the ability of non-pathogenic bacteria to induce intestinal epithelial pathophysiology *via* monocyte/ macrophage activation.

8. This project has provided additional evidence on the importance of the pro-inflammatory cytokine, TNFα, in the epithelial pathophysiology similar to that seen in Crohn's disease. We have shown increased production of TNFα by PBM activated by bacteria/bacterial products as well as LPMC from Crohn's disease mucosa. Our studies suggest that TNFα is a key factor mediating the monocyte/macrophage-induced epithelial pathophysiology, although not affecting the epithelium directly. TNFα affects the PBM *via* an autocrine pathway, leading to production of other, as yet unidentified, mediators which in turn cause epithelial pathophysiology. We have also shown that

neutralization of TNFα by a monoclonal anti-TNFα antibody inhibits much of the pathogenesis induced by PBM activated by bacteria/bacterial products.

9. Utilizing our simplified model of monocyte/macrophage-induced intestinal inflammation, we have clearly shown for the first time that the new analogs of the topical steroid, budesonide, particularly D5519, inhibit epithelial abnormalities more effectively than budesonide itself. The ability of these steroids to inhibit the PBM-mediated epithelial pathophysiology correlates with their ability to inhibit TNFα production, emphasizing the importance of this cytokine in mediating epithelial abnormalities.

We speculate that during times of transient intestinal injury and/or in the presence of a primary abnormality of the epithelial permeability, bacterial products normally present in the lumen leak through the epithelial barrier. In a healthy individual, the mucosal macrophages are tolerant towards the luminal bacteria and either no inflammation occurs or it is short lived. In a susceptible individual, however, this tolerance is lost, perhaps due to presence of $CD14^+$ macrophages which are sensitive to bacterial stimulation (Duchmann *et al.,* 1996; Duchmann *et al.,* 1995). Activation of these cells results in a cycle with a greater defect in the mucosal barrier and greater uptake of luminal material. We postulate that release of large quantities of proinflammatory mediators and cytokines such as TNFα from

macrophages can lead to downstream amplification of other immune responses and release of tissue damaging substances, the end result of which is the severe mucosal damage seen in inflammatory conditions of the gut such as Crohn's disease.

APENDIX

Expanded Description of Selected Methods

T84 CELLS AS A MODEL SYSTEM

The T84 cell line is a human colon crypt-like cell line that was first established by Dharsathaphorn et al., 1984. The cells are derived from a human colonic carcinoma and provide a well established model system for investigating intestinal epithelial function (Barrett, 1993). T84 cells grow as a monolayer on permeable filter support and develop as polarized epithelial cells with distinct apical and basolateral membranes with intracellular tight junctions. These cells display vectorial ion secretion which provide a measure of the secretory responses of the epithelium, and they respond like intact colon to secretagogues. In addition, the T84 cells attain high transepithelial electrical resistance which provides a measure of the barrier function of the epithelium. These characteristics enables the researcher to mount the monolayers in Ussing chambers, where functional data can be obtained to study the epithelial physiology and pathophysiology. This makes the T84 cell line an ideal model to study the intestinal epithelium under various experimental conditions.

Electrophysiology of an epithelium as measured by Ussing chambers

The Ussing chamber is an instrument designed to study active transport mechanisms and barrier functions of an epithelium . The apparatus consists of two reservoirs separated by a T84 monolayer or a piece of isolated intestine. The luminal (or apical) surface of the epithelium is bathed by the fluid of one chamber

while the serosal (or basolateral) surface is bathed by the other chamber. In order to eliminate chemical gradients from one chamber to the other, a voltage clamp apparatus provides sufficient current to nullify the spontaneous potential difference generated by the epithelium (Ussing and Zerahn, 1951). The current required to nullify the potential difference is called the short circuit current (Isc) and reflects the net amount of electrical charge actively transported by the epithelium. In T84 cells, the only ion to be actively transported by the epithelium is chloride; therefore the Isc provides an indication of chloride secretion in these cells (Barrett, 1993).

By applying Ohm's law, V=IR, the electrical resistance (R) can be calculated from the spontaneous potential difference (V) and an imposed current (I) (Karnaky, Jr. 1992). Electrical resistance is an indication of the barrier function of an epithelium (Powell, 1981). High electrical resistance values, like those obtained from T84 monolayers, indicate a "tight" epithelium with intact tight junctions between the cells (Barrett, 1993). Low electrical resistance indicates decreased barrier function with increased movement of ions and small molecules through the paracellular space (Madara and Trier, 1994).

REFERENCES

1. ALLISON, M. C. AND POULTER, L. W. Changes in phenotypically distinct mucosal macrophage populations may be a prerequisite for the development of inflammatory bowel disease. Clin. Exp. Immunol. **85**: 504-509, 1991.

2. ALPERS, D. H. Digestion and absorption of carbohydrates and proteins. In Physiology of the gastrointestinal tract, ed. by L. R. Johnson, D. H. Alpers, J. Christensen, E. D. Jacobson and J. H. Walsh, pp. 1723-1750, Raven Press, New York, NY, USA, 1994.

3. AMERONGEN, H. M., WELTZIN, R., MACK, J. A., WINNER, S., MICHETTI, P., APTER, F. M., KRAEHENBUHL, J. P. AND NEUTRA, M. R. M cell-mediated antigen transport and monoclonal IgA antibodies for mucosal immune protection. Ann. N. Y. Acad. Sci. **664**: 18-26, 1992.

4. BACHMAN, B. J. Derivation and genotype of some mutant derivatives of *Escherichia coli* K12. In *Escerichia coli* and *Salmonella ryphimurium*, ed. by F. C. Neidhardt, J. L. Ingraham, K. E. Low, B. Magasanik, M. Schacchter and H. F. Umbarger, pp. 1190-1219, American Society for Microbiology, Washington DC, 1987.

5. BALDASSANO, R. N., SCHREIBER, S., JOHNSTON, R. B., FU, R. D., MURAKI, T. AND MACDERMOTT, R. P. Crohn's disease monocytes are primed for accentuated release of toxic oxygen metabolites. Gastroenterology **105**: 60-66, 1993.

6. BARRETT, K. Positive and negative regulation of chloride secretion in T84 cells. Am. J. Physiol. **265**: C859-C868, 1993.

7. BARRETT, K. E. Immune-related intestinal chloride secretion: Acute and chronic effects of mast cell mediators on chloride secretion by a human colonic epithelial cell line. J. Immunol. **147**: 959-964, 1991.

8. BINDER, V. Progress in epidemiology, quality of life and life expectancy in IBD. In Inflammatory bowel diseases, ed. by G. N. J. Tytgat, J. F. W. A. Bartelsman and S. J. H. Van Deventer, pp. 27-31, Kluwer, Lancaster, 1995.

9. BRAEGGER, C. D., NICHOLLS, S., MURCH, S. H., STEPHENS, S. AND MACDONALD, T. T. Tumor necrosis factor α in stool as a marker of

intestinal inflammation. Lancet **339**: 89-91, 1992.

10. BRATTSAND, R. Overview of newer glucocorticosteroid preparations for inflammatory bowel disease. Can. J. Gastroenterol. **4**: 407-414, 1990.

11. BREESE, E. J., MICHIE, C. A., NICHOLLS, S. W., MURCH, S. H., WILLIAMS, C. B. AND DOMIZIO, P. Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. Gastroenterology **106**: 1455-1466, 1994.

12. BULL, D. M. AND BOOKMAN, M. A. solation and functional characterisation of human intestinal mucosal lymphoid cells. J. Clin. Invest. **59**: 966-974, 1977.

13. CAMPIERI, M., FERGUSON, A., DOE, W., PERSSON, T., NILSSON, L. AND AND THE GLOBAL BUDESONIDE STUDY GROUP: Oral budesonide is as effective as oral prednisolone in active Crohn's disease. Gut **41**: 209-214, 1997.

14. CAPPELLO, M., KESHAV, S., PRINCE, C., JEWELL, D. P. AND GORDON, S. Detection of mRNAs for macrophage products in inflammatory bowel disease by in situ hybridisation. Gut **33**: 1214-1219, 1992.

15. CHADWICK, V. S. AND ANDERSON, R. P. Inflammatory products of commensal bacteria and gastrointestinal disorders. Dig. Dis. **8**: 253-268, 1990.

16. CHANG, E. B. AND RAO, M. C. Intestinal water and electrolyte transport: Mechanisms of physiological and adaptive responses. In Physiology of the gastrointestinal tract, ed. by L. R. Johnson, D. H. Alpers, J. Christensen, E. D. Jacobson and J. H. Walsh, pp. 2027-2082, Raven Press, New York, NY, USA, 1994.

17. COMPEAU, C. G., ROTSTEIN, O. D., TOHDA, H., MARUNAKA, Y., RAFII, B., SLUTSKY, A. S. AND O'BRODOWICH, H. Endotoxin-stimulated alveolar macrophages impair lung epithelial Na^+ transport by an L-Arg-dependent mechanism. Am. J. Physiol. **266**: C1330-C1341, 1994.

18. COMPSTON, J. E., JUDD, D. AND CROWLEY, E. O. Osteoporosis in patients with inflammatory bowel disease. Gut **28**: 410-415, 1987.

19. CONE, R. A. Mucus. In Mucosal immunology, ed. by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock and J. R. McGhee, pp. 43-64, Academic Press, San Diego, CA, USA, 1999.

20. CONTRACTOR, N. V., BASSIRI, H., REYA, T., PARK, A. Y., BAUMGART, D. C., WASIK, M. A., EMERSON, S. G. AND CARDING, S. R. Lymphoid hyperplasia, autoimmunity, and compromised intestinal intraepithelial lymphocyte development in colitis-free gnobiotic IL-2 deficient mice. J. Immunol. **160**: 385-394, 1998.

21. CUI, N., FRIEND, D. R. AND FEDORAK, R. N. A budesonide prodrug accelerates treatment of colitis in rats. Gut **35**: 1439-1446, 1994.

22. DENTENER, M. A., BAZIL, V. AND VON ASMUTH, E. J. U. Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-α, IL-6 and IL-8 release by human monocytes and alveolar macrophages. J. Immunol. **150**: 2885-2891, 1993.

23. DIANDA, L., HANBY, A. M., WRIGHT, N. A., SEBESTENY, A., HAYDAY, A. C. AND OWEN, M. J. T cell receptor-αβ-deficient mice fail to develop colitis in the absence of a microbial environment. Am. J. Pathol. **150**: 91-97, 1997.

24. DUCHMANN, R., KAISER, I., HERMANN, E., MAYET, W., EWE, K. AND MEYER ZUM, K. H. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin. Exp. Immunol. **102**: 448-455, 1995.

25. DUCHMANN, R., SCHMITT, E., KNOLLE, P., BUSCHENFELDE, K. M. Z. AND NEURATH, M. Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. Eur. J. Immunol. **26**: 934-938, 1996.

26. DUCHMANN, R. AND ZEITZ, M. Crohn's disease. In Mucosal immunology, ed. by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock and J. R. McGhee, pp. 1055-1080, Academic Press, San Diego, CA, USA, 1999.

27. EKBOM, A., HELMICK, C., ZACK, M. AND ADAMI, H. O. Increased risk of large bowel cancer in Crohn's disease with colonic involvement. Lancet **336**: 357-359, 1990.

28. EKBOM, A., HELMICK, C., ZACK, M., HOLMBERG, L. AND ADAMI, H. O. Survival and causes of death in patients with inflammatory bowel disease: a population-based study. Gastroenterology **103**: 954-960, 1992.

29. FARSTAD, I. N., HALSTENSEN, T. S., FAUSA, O. AND BRANDTZAEG, P. Heterogeneity of M cell-associated B and T cells in human Peyer's patches. Immunology **83**: 457-464, 1994.

30. FELDMANN, M., BRENNAN, F. M., ELLIOTT, M. J., WILLIAMS, R. O. AND MAINI, R. N. TNF alpha is an effective therapeutic target for rheumatoid arthritis. Ann. NY. Acad. Sci. **766**: 272-278, 1995.

31. FREY, A., LENCER, W. I., WELTZIN, R., GIANNASCA, K. T., GIANNASCA, P. J. AND NEUTRA, M. R. Role of glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. J. Exp. Med. **184**: 1045-1060, 1996.

32. GARDINER, K. R., HALLIDAY, M. I., BARCLAY, G. R., MILNE, L., BROWN, D., STEPHENS, S., MAXWELL, R. J. AND ROWLANDS, B. J. Significance of systemic endotoxaemia in inflammatory bowel disease. Gut **36**: 897-901, 1995.

33. GOODLAD, R. A. Gastrointestinal epithelial cell proliferation. Dig. Dis. **7**: 169-177, 1989.

34. GRANT, J. T. AND HUSBY, G. Joint manifestations in gastrointestinal diseases. 1. Pathophysiological aspects, ulcerative colitis and Crohn's disease. Dig. Dis. **10**: 274-294, 1992.

35. GREENBERG, G. R., FEAGAN, B. G., MARTIN, F., SUTHERLAND, L. R., THOMSON, A. B. R., WILLIAMS, C. N., NILSSON, L. AND PERSSON, T. Oral budesonide for active Crohn's disease. N. Engl. J. Med. **331**: 836-841, 1994.

36. GREENSTEIN, A. J., JANOWITZ, H. D. AND SACHAR, D. B. The extra-intestinal complications of Crohn's disease and ulcerative colitis: A study of 700 patients. Medicine **55**: 401-412, 1976.

37. GRIMM, M. C., PAVLI, P., VAN DE POL, E. AND DOE, W. F. Evidence for a $CD14^+$ population of monocytes in inflammatory bowel disease mucosa;

implications for pathogenesis. Clin. Exp. Immunol. **100**: 291-297, 1995a.

38. GRIMM, M. C., PULLMAN, W. E., BENNETT, G. M., SULLIVAN, P. J., PAVLI, P. AND DOE, W. F. Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. J. Gastroenterol. Hepatol. **10**: 387-395, 1995b.

39. GUSTAFSSON, J. A., CARLSTEDT-DUKE, J. AND STROMSTEDT, P. E. Structure, function and regulation of the glucocorticoid receptor. In Molecular endocrinology and steroid hormone action, ed. by G. H. Sato and J. L. Stevens, pp. 65-82, Allan R Liss, New York,NY, USA, 1990.

40. HEYMAN, M., DCROC, R., DESJEUX, J. F. AND MORGAT, J. L. Horseradish peroxidase transport across adult rabbit jejunum *in vitro*. Am. J. Physiol. **242**: G558-G564, 1982.

41. HEYMAN, M., CRAIN-DENOYELLE, A. M., NATH, S. K. AND DESJEUX, J. Quantification of protein transcytosis in the human colon carcinoma cell line CaCo-2. J. Cell Physiol. **143**: 391-395, 1990.

42. JAKOBOVITS, J. AND SCHUSTER, M. M. Metronidazole therapy for crohn's disease and associated fistulae. Am. J. Gastroenterol. **79**: 533-540, 1984.

43. JONES, B. D., GHORI, N. AND FALKOW, S. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patch. J. Exp. Med. **180**: 15-23, 1994.

44. JWELL, D. AND RUTGEERTS, P. Reviewing steroids in the treatment of inflammatory bowel disease. Res. Clin. Forum **15**: 1-103, 1993.

45. KARNAKY, K. J.,JR. Electrophisiological assessment of epithelia. In Cell-Cell Interactions, ed. by B. R. Stevenson, W. J. Gallin and D. L. Paul, pp. 257-274, Oxford, UK:IRL, 1992.

46. KATO, T. AND OWEN, R. L. Structure and function of intestinal mucosal epithelium. In Mucosal immunology, ed. by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock and J. R. McGhee, pp. 115-132, Academic Press, San Diego, CA, USA, 1999.

47. KAUNITZ, J. D., BARRETT, K. E. AND MCROBERTS, J. A. Electrolyte secretion and absorption: small intestine and colon. In Textbook of

gastroenterology, ed. by J. B. Yamada, pp. 326-361, Lippincott Co. Philadelphia, PA, USA, 1994.

48. KLASEN, I. S., MELIEF, M. J. AND VAN HALTEREN, A. G. S. The presence of peptidoglycan-polysaccharide complexes in the bowel wall and the cellular responses to these complexes in Crohn's disease. Clin. Immunol. Immunopathol. **71**: 303-308, 1994.

49. KUHN, R., LOHLER, J., RENNICK, D., RAJEWSKY, K. AND MULLER, W. Interleukin-10 deficient mice develop chronic entrocolitis. Cell **75**: 263-274, 1993.

50. KUSUGAMI, K., FUKATSU, A., TANIMOTO, M., SHINODA, M., HARUTA, J., KUROIWA, A., INA, K., KANAYAMA, K., ANDO, T. AND SARAI, S. Elevation of interleukin-6 in Crohn's disease is macrophage- and epithelial cell-dependent. Dig. Dis. Sci. **40**: 949-959, 1995.

51. LINDEN, M. AND BRATTSAND, R. Effects of a corticosteroid, budesonide, on alveolar macrophage and blood monocyte secretion of cytokines: differential sensitivity of GM-CSF, IL-1β and IL-6. Pulmonary Pharmacol. **7**: 43-47, 1994.

52. LOFBERG, R., DANIELSSON, A. AND SALDE, L. Oral budesonide in active Crohn's disease. Aliment. Pharmacol. Ther. **7**: 611-616, 1993.

53. LOFBERG, R., DANIELSSON, A., SUHR, O., NILSSON, A., SCHIOLER, R., NYBERG, A., HULTCRANTZ, R., KOLLBERG, B., GILLBERG, R., WILLEN, R. AND PERSSON, T. Oral budesonide versus prednisolone in patients with active extensive and left-sided ulcerative colitis. Gastroenterology **110**: 1713-1718, 1996.

54. LORD, P. C. W., WILMOTH, L. M. G. AND MIZEL, S. B. Expression of interleukin-1α and β genes by human blood polymorphonuclear leukocytes. J. Clin. Invest. **87**: 1312-1321, 1991.

55. LUGERING, N., KUCHARZIK, T., STOLL, R. AND DOMSCHKE, W. Current concept of the role of monocyte/macrophages in inflammatory bowel disease -Balance of proinflammatory and immunosuppressive mediators. Ital. J. Gastroenterol. Hepatol. **30**: 338-344, 1998.

56. LUNDQVIST, C., MELGAR, S., YEUNG, M. M., HAMMARSTROM, S. AND

HAMMARSTROM, M. L. Intaepithelial lumphocytes in human gut have lytic potential and a cytokine profile that suggest Th1 and cytotoxic functions. J. Immunol. **157**: 1926-1934, 1996.

57. MACDONALD, T. T., HUTCHINGS, P., CHOY, M., MURCH, S. H. AND COOKE, A. Tumor necrosis factor α and interferon gamma production measured at the single cell level in normal and inflamed human intestine. Clin. Exp. Immunol. **81**: 301-305, 1990.

58. MACDONALD, T. T. Breakdown of tolerance to the intestinal bacterial flora in inflammatory bowel disease (IBD). Clin. Exp. Immunol. **102**: 445-447, 1995.

59. MACDONALD, T. T. AND SPENCER, J. Evidence that activated mucosal T cells play a role in the pathogenesis of enteropathy in human small intestine. J. Exp. Med. **167**: 1341-1349, 1988.

60. MADARA, J. L., PATAPOFF, T. W., GILLECE-CASTRO, B., COLGAN, S. P., PARKOS, C. A., DELP-ARCHER, C. AND MRSNY, R. J. 5'-Adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. J. Clin. Invest. **91**: 2320-2325, 1993.

61. MADARA, J. L. AND STAFFORD, J. Interferon-gama directly affects barrier function of cultured intestinal epithelial monolayers. J. Clin. Invest. **83**: 724-727, 1989.

62. MADARA, J. L. AND TRIER, J. S. The functional morphology of the mucosa of the small intestine. In Physiology of the gastrointestinal tract, ed. by J. B. Yamada, pp. 1577-1622, Raven Press, New York, NY, USA, 1994.

63. MADSEN, K. L., LEWIS, S. A., TAVERNINI, M. M., HIBBARD, J. AND FEDORAK, R. N. Interleukin 10 provides cytokine-induced disruption of T84 monolayer barrier integrity and limits chloride secretion. Gastroenterology **113**: 151-159, 1997.

64. MAHIDA, Y. R., PATEL, S., GIONCHETTI, P., VAUX, D. AND JEWELL, D. P. Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. Gut **30**: 826-834, 1989a.

65. MAHIDA, Y. R., WU, K. C. AND JEWELL, D. P. Respiratory burst activity of

intestinal macrophages in normal and inflammatory bowel disease. Gut **30**: 1362-1370, 1989b.

66. MAHIDA, Y. R., KURLAK, L., GALLAGHER, A. AND HAWKEY, C. J. High circulating levels of interleukin-6 in active Crohn's disease but not ulcerative colitis. Gut **32**: 1531-1534, 1991.

67. MALIZIA, G., CALABRESE, A., COTTONE, M., RAIMONDO, M., TREJDOSIEWICZ, L. K., SMART, C. J., OLIVA, L. AND PAGLIANO, L. Expression on leukocyte adhesion molecules by mucosal mononuclear phagocytes in inflammatory bowel disease. Gastroenterology **100**: 150-159, 1991.

68. MAURY, J., NICOLETTI, C., GUZZO-CHAMBRAUD, L. AND MAROUX, S. The filamentous brush border glycocalyx, a mucin-like marker of enterocyte hyper-polarization. Eur. J. Biochem. **228**: 323-331, 1995.

69. MCKAY, D. M., CROITORU, K. AND PERDUE, M. H. T cell-monocyte interactions regulate epithelial physiology in a coculture model of inflammation. Am. J. Physiol **270**: C418-C428, 1996.

70. MONTELEONE, G., BIANCONE, L., MARASCO, R., MORRONE, G., MARASCO, O., LUZZA, F. AND PALLONE, F. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. Gastroenterology **112**: 1169-1178, 1997.

71. MORISE, K., YAMAGUCHI, T., KUROIWA, A., KANAYAMA, K., MATSUURA, T., SHINODA, M., YAMAMOTO, H., HORIUCHI, Y., FURUSAWA, A., IWASE, H., KUSUGAMI, K. AND NAGURA, H. Expression of adhesion molecules and HLA-DR by macrophages and dendritic cells in aphtoid lesions of Crohn's disease: an immunohistochemical study. J. Gastroenterol. **29**: 257-264, 1994.

72. MOSLEY, R. L., WHETSELL, M. AND KLEIN, J. R. Proliferative properties of murine intraepithelial lymphocytes (IEL): IEL expressing TcR $\alpha\beta$ or TcR g are largely unresponsive to proliferative signals mediated via conventional stimulation of the CD3-TcR complex. Int. Immunol. **3**: 563-569, 1991.

73. NAGASHIMA, R., MAEDA, K., IMAI, Y. AND TAKAHASHI, T. Lamina propria macrophages in the human gastrointestinal mucosa: their distribution, immunohistological phenotype and function. J. Histochem. Cytochem. **44**:

721-731, 1996.

74. NAST, C. C. AND LEDUC, L. E. Chemotactic peptides: mechanisms, functions, and possible role in inflammatory bowel diseases. Dig. Dis. Sci. **33**: 50S-57S, 1988.

75. NATHAN, C. F. Secretory products of macrophages. J. Clin. Invest **79**: 319-326, 1987.

76. NEUTRA, M. R., FREY, A. AND KRAEHENBUHL, J. P. Epithelial M cells: gateways for mucosal infection and immunization. Cell **86**: 345-348, 1996.

77. NEUTRA, M. R. Current concepts in mucosal immunity: V. Role of M cells in transepithelial transport of antigens and pathogens to the mucosal immune system. Am. J. Physiol. **274**: G785-G791, 1998.

78. ODDERA, S., SILVESTRI, M., SACCO, O., LANTERO, S., MORELLI, M. C. AND ROSSI, G. Evaluation of the inhibitory effects of budesonide on the mitogen-induced or the allergen-induced activation of blood mononuclear cells isolated from asthmatic patients. Ann. Allergy Asthma Immunol. **75**: 33-40, 1995.

79. OUELLETTE, A. J. Paneth cells and innate imunity in the crypt microenvironment. Gastroenterology **113**: 1779-1784, 1997.

80. OUELLETTE, A. J. AND SELSTED, M. E. Paneth cell defensins: endogenous peptide components of intestinal host defense. FASEB J. **10**: 1280-1289, 1996.

81. PAVLI, P. AND DOE, W. F. Intestinal macrophages. In Inflammatory bowel disease, ed. by R. P. MacDermott and W. F. Stenson, pp. 177-188, Elsevier, New York, NY, USA, 1992.

82. PEDERSON, S. AND O'BYRNE, P. A comparison of the efficacy and safety of inhaled corticosteroid in asthma. Eur. J. Allergy Clin. Immunol. **52**: 1-34, 1997.

83. PEPPERCORN, M. A. Is there a role for antibiotics as primary therapy in Crohn's ileitis? J. Clin. Gastroenterol. 235-237, 1993.

84. PERDUE, M. H. AND MCKAY, D. M. Integrative immunophysiology in the

intestinal mucosa. Am. J. Physiol. **267**: G151-G165, 1994.

85. PERDUE, M. H. AND MCKAY, D. M. Mucosal regulators and immunopathophysiology. Curr. Opin. Gastroentrology **12**: 591-599, 1996.

86. PERSSON, P. G., BERNELL, O., LEIJONMARK, C. E., FARAHMAND, B. Y., HELLERS, G. AND AHLBOM, A. Survival and cause-specific mortality in inflammatory bowel disease: a population-based cohort study. Gastroenterology **110**: 1339-1345, 1996.

87. PHILPOTT, D. J., MCKAY, D. M., SHERMAN, P. M. AND PERDUE, M. H. Infection of T84 cells with enteropathogenic *Escherchia coli* alters barrier and transport functions. Am. J. Physiol. **270**: G634-G645, 1996.

88. PIRZER, U. C., SCHUURMAN, G., POST, S., BETZLER, M. AND MEUER, S. C. Differential responsiveness to CD3 Ti vs CD2-dependent activation ofhuman intestinal T lymphocytes. Eur. J. Immunol. **20**: 2339-2344, 1990.

89. PLEVY, S. E., LANDERS, C. J., PREHN, J., CARRAMANZANA, N. M., DEEM, R. L., SHEALY, D. AND TARGAN, S. R. A role for TNFα and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. J. Immunol. **159**: 6276-6282, 1997.

90. POWELL, D. W. Barrier function of epithelia. Am. J. Physiol. **241**: G275-G288, 1981.

91. RATH, H. C., HERFARTH, H. H., IKEDA, J. S., GRENTHER, W. B., HAMM, T. E., BALISH, E., TAUROG, J. D., HAMMER, R. E., WILSON, K. H. AND SARTOR, R. B. Normal luminal bacteria, specially *Bacteroides* species, mediate chronic colitis, gastritis and arthritis in HLA-B27/Human β_2 microglobulin transgenic rats. J. Clin. Invest. **98**: 945-953, 1996.

92. REIMUND, J., WITTERSHEIM, C., DUMONT, S., MULLER, C. D., KENNEY, J. S., BAUMANN, R., POINDRON, P. AND DUCLOS, B. Increased production of tumor necrosis factor-α, interleukin-1β, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. Gut **39**: 684-689, 1996.

93. REINECKER, H. C., STEFFEN, M., WITTHOEFT, T., PFLUEGER, I., SCHREIBER, S., MACDERMOTT, R. P. AND RAEDLER, A. Enhanced secretion of tumor necrosis factor α, IL-6, and IL-1 β by isolated lamina

propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clin. Exp. Immunol. **94**: 174-181, 1993.

94. REINECKER, H. C., LOH, E. Y., RINGLER, D. J., MEHTA, A., ROMBEAU, J. A. AND MACDERMOTT, R. P. Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. Gastroenterology **108**: 40-50, 1995.

95. RODRIGUEZ, P., HEYMAN, M., CANDALH, C., BLATON, M. A. AND BOUCHAUD, C. Tumor necrosis factor-α induces morphological and functional alterations of intestinal HT29 cl.19A cell monolayers. Cytokine **7**: 441-448, 1995.

96. ROGLER, G., HAUSMANN, M., VOGL, D., ASCHENBRENNER, E., ANDUS, T., FALK, W., ANDREESEN, R., SCHOLMERICH, J. AND GROSS, V. Isolation and phenotypic characterization of colonic macrophages. Clin. Exp. Immunol. **112**: 205-215, 1998.

97. RUGTVEIT, J., BRANDTZAEG, P., HALSTENSEN, T. S., FAUSA, O. AND SCOTT, H. Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes. Gut **35**: 669-674, 1994.

98. RUGTVEIT, J., HARALDSEN, G., HOGASEN, A. K., BAKKA, A., BRANDTZAEG, P. AND SCOTT, H. Respiratory burst of intestinal macrophages in inflammatory bowel disease is mainly caused by CD14+ L1+ monocyte derived cells. Gut **37**: 367-373, 1995.

99. RUGTVEIT, J., NILSEN, E. M., BAKKA, A., CARLSEN, H., BRANDTZAEG, P. AND SCOTT, H. Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. Gastroenterology **112**: 1493-1505, 1997.

100. RUTGEERTS, P., GEBOES, K., PEETERS, M., HIELE, M., PENNINCKX, F., AERTS, R., KERREMANS, R. AND VANTRAPPEN, G. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal illeum. Lancet **338**: 771-774, 1991.

101. RUTGEERTS, P., LOFBERG, R., MALCHOW, H., LAMERS, C., OLAISON, G., JWELL, D., DANIELSSON, A., GOEBELL, H., OSTERGARD THOMPSEN, O., LORENZ-MEYER, H., HODGSON, H., PERSSON, T. AND

SEIDEGAARD, C. A comparison of budesonide with prednisolone for active Crohn's disease. N. Engl. J. Med. **331**: 842-845, 1994.

102. RUTGEERTS, P., D'HAENS, G., TARGAN, S., VASILIAUSKAS, E., HANAUER, S. B., PRESENT, D. H., MAYER, L., VAN HOGEZAND, R. A., BRAAKMAN, T. A. J., DEWOODY, K. L., SCHAIBLE, T. F. AND VANDEVENTER, S. J. H. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (Infliximab) to maintain remission in Crohn's disease. Gastroenterology **117**: 761-769, 1999.

103. SADLACK, B., MARZ, H., SCHORLE, H., SCHIMPL, A., FELLER, A. C. AND HORAK, I. Ulcerative colitis like disease in mice with a disrupted interleukin-2 gene. Cell **75**: 253-261, 1993.

104. SARTOR, R. B. The role of enteric microflora in the pathogenesis of intestinal inflammation and arthritis. Aliment. Pharmacol. Ther. **11**: 17-23, 1997.

105. SCHREIBER, S., HEINIG, T., THIELE, H. AND RAEDLER, A. Immunoregulatory role of interleukin 10 in patients in inflammatory bowel disease. Gastroenterology **108**: 1434-1444, 1995.

106. SELLON, R. K., TONKONOGY, S. L., SCHULTZ, M., DIELEMAN, L. A., GRENTHER, W. B., BALISH, E., RENNICK, D. AND SARTOR, R. B. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10 deficient mice. Infect. Immun. **66**: 5224-5231, 1998.

107. SHANAHAN, F. The intestinal immune system. In Physiology of the Gastrointestinal Tract, ed. by L. R. Johnson, pp. 643-684, Raven Press, New York, NY, USA, 1994.

108. SHERRIFF, R. M., BROOM, M. F. AND CHADWICK, V. S. Isolation and purification of N-formylmethionine aminopeptidase from rat intestine. Biochem. Biophys. Acta **1119**: 275-280, 1992.

109. SINGLETON, J. W., LAW, D. H. AND KELLY, M. L. J. ational cooperative Crohn's disease study: adverse reactions to study drugs. Gastroenterology **77**: 870-882, 1979.

110. STEVENS, C., WALZ, G., SINGARAM, C., LIPMAN, M. L., ZANKER, B.,

MUGGIA, A., ANTONIOLI, D., PEPPERCORN, M. A. AND STROM, T. B. Tumor necrosis factor-α, interleukin-1β, and interleukin-6 expression in inflammatory bowel disease. Dig. Dis. Sci. **37**: 818-826, 1992.

111. SURETTE, M. E., PALMANTIER, R. AND GOSSELIN, J. Lipopolysaccharides prime whole human blood and isolated neutrophils for the increased synthesis of 5-lipoxugenase products by enhancing arachidonic acid availability-involvment of the CD14 antigen. J. Exp. Med. **178**: 1347-1355, 1993.

112. SUTHERLAND, L. R., SINGLETON, J. W., SESSIONS, J., HANAUER, S. B., KRAWITT, E., RANKIN, G., SUMMERS, R., MEKHJIAN, H., GRENBERGER, N., KELLY, M., LEVINE, J., THOMSON, A., ALPERT, E. AND PROKIPCHUK, E. Double blind, placebo controlled trial of metronidazole in Crohn's disease. Gut **32**: 1071-1075, 1991.

113. TAGUCHI, T., MCGHEE, J. R., COFFMAN, R. L., BEAGLEY, K. W., ELDRIDGE, J. H., TAKATSU, K. AND KIYONO, H. Analysis of Th1 and Th2 in murine gut-associated tissues. Frequencies of CD4+ and CD8+ T cells that secrete IFN-g and IL-5. J. Immunol. **145**: 68-77, 1990.

114. TARGAN, S., DEEM, R. L., LIU, M., WANG, S. AND NEL, A. Definition of a lamina propria T cell responsive state. Enhanced cytokine responsiveness of T cells stimulated through the CD2 pathway. J. Immunol. **154**: 664-675, 1995.

115. TARGAN, S. R., HANAUER, S. B., VANDEVENTER, S. J. H., MAYER, L., PRESENT, D. H., BRAAKMAN, T. A. J., DEWOODY, K. L., SCHAIBLE, T. F. AND RUTGEERTS, P. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor α for crohn's disease. N Engl J Med **337**: 1029-1035, 1997.

116. TAYLOR, C. T., MURPHY, A., KELLEHER, D. AND BAIRD, A. W. Changes in barrier function of a model intestinal epithelium by intraepithelial lymphocytes require new protein synthesis by epithelial cells. Gut **40**: 634-640, 1997.

117. TAYLOR, C. T., DZUS, A. L. AND COLGAN, S. P. Autocrine regulation of epithelial permeability by hypoxia: role for polarized release of tumor necrosis factor α. Gastroenterology **114**: 657-668, 1998.

118. THALEN, B. A., AXELSSON, B. I., ANDERSSON, P. H., BRATTSAND, R. L., NYLANDER, B. AND WICKSTORM, L. 6α-Fluoro- and 6α,9α-difluoro-11β,21-dihydroxy-16α,17α-propylmethylene-dioxypregn-4-ene-3,20-dione: synthesis and evaluation of activity and kinetics of their C-22 epimers. Steroids **63**: 37-43, 1998.

119. THOMPSON, C. B. Distinc roles for the costimulatory ligands B7-1 and B7-2 in T-helper cell differentiation. Cell **81**: 979-982, 1995.

120. USSING, H. H. AND ZERAHN, K. Active transport of sodium as the source of electric current in the short-circuited frog skin. Acta Physiol. Scand. **23**: 110-127, 1951.

121. VAN DULLEMENT, H. M., VANDEVENTER, S. J. H., HOMMES, D. W., BIJL, H. L., JANSEN, J., TYTGAT, G. N. AND WOODY, J. Treatment of crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology **109**: 129-135, 1995.

122. VANDEVENTER, S. J. H. Tumor necrosis factor and Crohn's disease. Gut **40**: 443-448, 1997.

123. WAAGE, A. AND BAKKE, O. Glucocorticoids suppress the production of tumor necrosis factor by lipopolysaccharide-stimulated human monocytes. Immunol. **63**: 299-302, 1988.

124. YAMAMOTO, M., FUJIHASHI, K., BAGLEY, K. W., MCGHEE, J. R. AND KIYONO, H. Cytokine synthesis by intestinal intraepithelial lymphocytes: Both g/ T-cells receptor-positive and a/b T-cell receptor-positive T-cells in the G(1)-phase of cell cycle produce IFNg and IL-5. J. Immunol. **150**: 106-114, 1993.

125. YOUNGMAN, K. R., SIMON, P. L., WEST, G. A., COMINELLI, F., RACHMILEWITZ, D., KLEIN, J. S. AND FIOCCHI, C. Localization of intestinal interleukin 1 activity, protein and gene expression to lamina propria cells. Gastroenterology **104**: 749-758, 1993.

126. ZAREIE, M., MCKAY, D. M., KOVARIK, G. G. AND PERDUE, M. H. Monocyte/macrophages evoke epithelial dysfunction: indirect role of tumor necrosis factor-α. Am. J. Physiol. **275**: C932-C939, 1998.