Influence of Natural Substances on Mediators of Intestinal Inflammation *in vitro*

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To my family

"O valor das coisas não está no tempo em que elas duram,
mas na intensidade com que acontecem.
Por isso existem momentos inesquecíveis,
coisas inexplicáveis e
pessoas incomparáveis: vocês".

Fernando Pessoa

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ABBREVIATIONS

ABCB1	ATP binding cassette B1 or multi-drug resistance (MDR)-1
ABCC2	ATP binding cassette C2 or multi-drug resistance associated protein (MRP)-2
ADAM	A disintegrin and metalloproteinase domain
AP-1	Activator protein-1
API	Apoptosis inhibitor
ATP	Adenosine triphosphate
BA	Boswellic acid
Bcl	B-cell CLL/lymphoma
BS	*Boswellia serrata*
CASP	Caspase
CD	Cluster and differentiation
CFb	Complement factor B
CLDN	Claudin
COL	collagen
COX	Cyclooxygenase
CSF	colony stimulating factor
DMSO	Dimethyl sulphoxide
DR	Death receptor
ECM	Extracellular matrix
EGCG	Epigallocatechin-3-gallate
FADD	Fas-related death domain protein
FCS	Foetal calf serum
FRA	Fos-related antigene
GBP	Guanylate binding protein
GCP	Granulocyte chemotactic protein
GJB	Gap junction protein beta
GRO	Growth regulated oncogene
GTP	Guanidine triphosphate
ICAM	Intercellular adhesion molecule
IEX-1L	Congenic gene of inhibitor apoptosis protein
IFI	IFN-γ-inducible protein
IFN-γ	Interferon-gamma
IFNGR	IFN-γ receptor
IFIT	IFN-inducible protein with tetratricopeptide repeats

IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
IRF	IFN-regulatory factor
β 5 integrin	Beta 5 integrin
IP	Interferon-inducible protein
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LAMA3	Laminin alpha 3
LAMB3	Laminin beta 3
LAMC2	Laminin gamma 2
5-LOX	5-lipoxygenase
MAPK	Mitogen-activated protein kinase
MCL1	Myeloid cell leukaemia sequence 1 (Bcl2-related)
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor-kappa B
NK4	Natural killer cell transcription-4
PDCD-6	Programmed cell death-6
PG	Prostaglandin
PGES	Prostaglandin E synthase
PI	Protease inhibitor
PI3K	Phosphatidylinositol 3-kinase
PKC	Phosphokinase C
PLAUR	Plasminogen activator, urokinase receptor
PML	Polymorphonuclear leukocytes
p62	Sequestosome 1
PTPase	Protein tyrosine phosphatase
RANK	Receptor activator of nuclear factor kappa B
RANTES	Regulated on activation normal T cell expressed and secreted
RIP	Receptor-interacting protein
RO	*Rosmarinus officinalis*
RT-PCR	Reverse transcription polymerase chain reaction
SDC4	Syndecan 4
STAT	Signal transducer and activator of transcription

TANK	TRAF family member-associated NF-κB activator
TNF-α	Tumor necrosis factor-alpha
TNFAIP	TNF-α-induced protein
TNFR	TNF receptor
TNIP	TNFAIP3 interacting protein 1
TRADD	TNFR-associated death domain protein
TRAF	TNFR-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAILR	TRAIL receptor
UC	Ulcerative colitis
XAF	XIAP-associated factor
XIAP	X-linked inhibitor of apoptosis

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INTRODUCTION

Prologue

The concept of an integrated and tightly regulated multicellular response in pathological processes in general, and inflammation in particular, has been applied to several organs and tissues, but only very recently this concept has been considered in the gut. There is a remarkable paucity of information on cellular interactions in complex inflammatory diseases, such as Crohn's disease (CD) and ulcerative colitis (UC), and there are essentially few data derived from animal models of inflammatory bowel disease (IBD). This makes it difficult to understand the cellular and molecular mechanisms underlying gut inflammation in human. Intestinal inflammation has traditionally been viewed as a process in which effector cells cause the destruction of other mucosal cells that behave as passive bystander targets (Fiocchi, 1997b).

According to the Crohn's and Colitis Foundation of America (CCFA), up to one million Americans suffer from IBD, with approximately 30 000 new cases diagnosed each year. Males and females appear to be affected equally. CD may occur in people of all ages, but it is primarily a disease of the young adult. Most cases are diagnosed before age 30, although a much smaller number of patients may develop the disease between the ages of 50 and 70 (http://www.ccfa.org).

Moreover, CD tends to occur in families and certain ethnic groups. Studies have shown that about 20 to 25 percent of patients may have a close relative with either CD or UC. It has been estimated that approximately five to eight percent of patients with CD may have a first-degree relative (brother, sister, parent, or child) with IBD. This does not seem to be any clear-cut pattern to this familial clustering, and the inheritance patterns are probably very complex. Researchers are actively attempting to establish a link to specific genes that may govern the transmission of the disease. The data suggest that more than one gene may be involved (Bonen and Cho, 2003).

At present, there is a lack of information on human gastrointestinal (GI) inflammatory diseases, regarding the specific genes involved. The present project aims to tackle this issue by finding new answers that may help in the treatment of individuals who are afflicted with or are at risk for GI inflammatory diseases.

Gastrointestinal physiology

General Functions of the Gastrointestinal (GI) Tract

The GI system provides the body with a continual supply of water, electrolytes, and nutrients. The process of food reduction occurs in five main phases: ingestion, fragmentation, digestion, absorption and elimination. To achieve these requirements, the alimentary tract exhibits typical layers for the better absorption of the nutrients. Figure 1 shows a cross section of the intestinal wall, including the layers from the outer surface inward: (1) the *serosa*, (2) the *longitudinal muscle layer*, (3) a *circular muscle layer*, (4) the *submucosa*, and (5) the *mucosa*.

The *mucosa* is divided in three layers: an epithelial layer, a connective tissue layer called *lamina propria,* and a fine smooth musculature layer, the *muscularis mucosae*, which causes local movements of the mucosa. The *submucosa* is a connective tissue layer that maintains the mucosa and which contains large blood vessels, lymphatic and nerves. The *muscular propria* is formed by smooth muscles that generally are subdivided in two histological layers: an *internal circular* layer and a *longitudinal external* layer. The action of these smooth muscular layers, placed in perpendicular angles to each other, is the bases of the peristaltic contraction.

The food passes through the GI tract by the action of two mechanisms: (1) the voluntary muscular action of the oral cavity, the *pharynx*, and the superior third of oesophagus (2) following of the appearance of involuntary contraction waves of the smooth musculature, the *peristaltic*. The latter and the secretory activity of all digestive tract are regulated by the autonomous nervous system and diverse hormones, some of which are secreted by the endocrines cells located within the own tract. Due to its continuity to the exterior, the GI tract is a potential entrance for pathogenic organisms. For this reason, the system incorporates a certain amount of defensive mechanisms between which are important lymphoid tissue aggregates distributed through the entire alimentary tract.

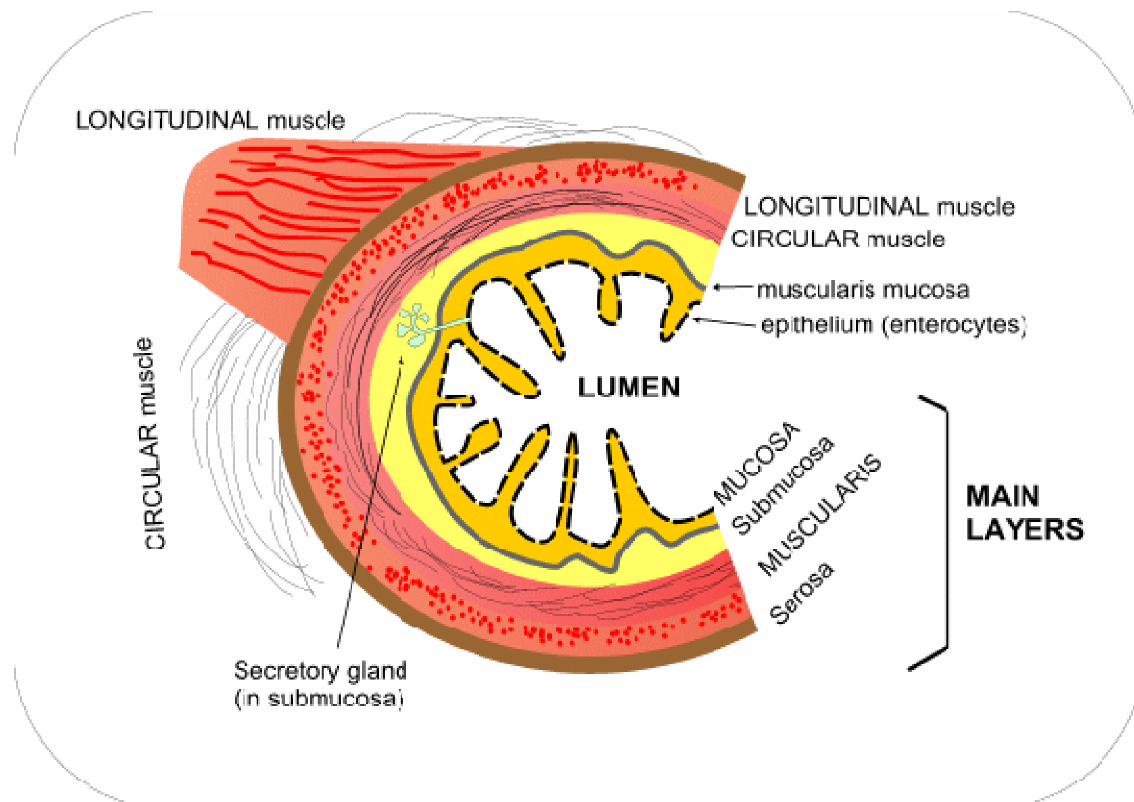


Figure 1: Typical cross section of the gut

(*http://medweb.bham.ac.uk/research/toescu/Teaching/GIT/StrucWall.gif*)

The "splanchnic circulation"

The *splanchnic circulation* includes the blood flow through the gut, spleen, pancreas, and liver. The blood that courses through the gut, spleen, and pancreas flows immediately into the liver by way of the portal vein. In the liver, the blood circulates through millions of sinusoids before leaving it by way of the hepatic veins that empty into the vena cava of the general circulation (Fig. 2). This is the secondary flow of the blood through the liver and it affords the reticuloendothelial cells that line the liver sinusoids to remove bacteria and other harmful agents that might enter the general blood stream from the GI tract.

The liver sinusoids also receive most of the non-fat, water-soluble nutrients absorbed from the gut. In addition, reticuloendothelial and hepatic cells absorb and store temporarily half to three quarters of all absorbed nutrients. The fat-based nutrients are absorbed into the intestinal lymphatics which are then conducted to the circulating blood by way of the thoracic duct, bypassing the liver.

Under normal conditions, the blood circulates in each area of the GI tract, including all layers of the gut wall. The local activity of the gut is directly related to the level of blood that flows into each intestinal vessel. For instance, after a meal, the motor, secretory and absorptive activities are increased; likewise the blood flow increases extremely but reduces to the resting level over another 2 to 4 hours. The blood flow in the villi and adjacent regions of

the submucosa is also increased during active absorption of nutrients (Vanner and Surprenant, 1996).

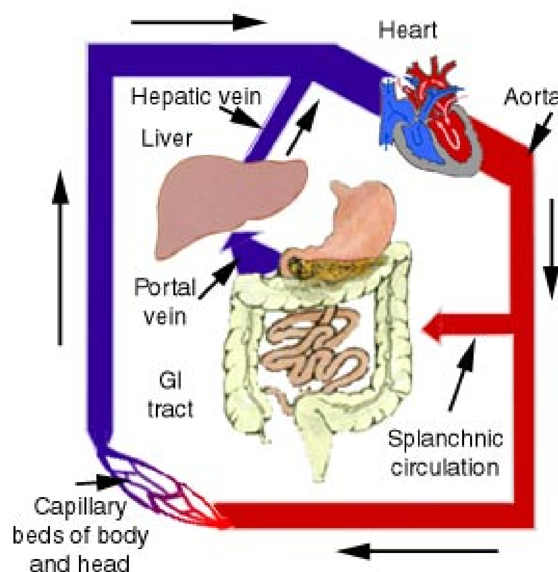


Figure 2: The gastrointestinal circulation
(*http://abdellab.sunderland.ac.uk/Lectures/Nurses/pics/anatomy/LiverBile.html*)

Features of the intestine

The small intestine is mainly involved in the absorption of nutrients via a vast surface, approximately four or six meters in length in a man. In addition, the disposition of the mucosa, forming numerous projections, called *villous* and the presence of invaginations between the bases of the villous towards the interior of the crypts (called *Lieberkuhn's crypts*) also increase the absorption (for review see Kaminsky and Zhang, 2003).

The intestinal villous are covered by a simple cylindrical epithelium which is continued with the epithelium of crypts. This epithelium is formed by the enterocytes, which are high cylindrical cells with a basal nucleus, and by the *caliciform cells*, which are distributed between the enterocytes. The intestinal epithelium renews completely every three-five days due to its continuous cells flaking that are located in the end of the villous, towards the intestinal light. The mitotic activity is made into the crypts in where the cells mature before degenerating and being eliminated in the end of the villous (Okamoto and Watanabe, 2004).

A third cellular type, that lacks digestive or absorbent properties, is located in the base of the crypts. These cells, called Paneth's cells, are loaded of intensely eosinophiles grains, and constitute a characteristic detail of the human small intestine, because in some mammals they do not exist. The Paneth's cells constitute a stable population and has ultra-structural characteristics of exocrine cells - secretors of proteins, although its complete function is not clarified.

Gastrointestinal Immunology

Intestinal epithelial cells and their function in the mucosal immune system

Intestinal epithelial cells are the first cells to come into contact with luminal antigens present in the intestinal microflora. They compose a barrier that separates the internal host from the external environment. Enterocytes also have an important role in fluid absorption and secretion as well as ion transport. These features of the intestinal epithelial cells are aided by intercellular tight junctions that restrict the passage of even small molecules (2 kDa) (Clayburgh et al., 2004). Moreover, epithelial cells at mucosal surfaces are an integral and essential component of the host's innate and acquired immune system. They constantly interact with invasive and non-invasive pathogens and adjacent and underlying cells in the mucosa by generating and transmitting signals. Thus, these cells constitutively express, or can be induced to express molecules involved in antigen presentation such as HLA class I and class II molecules (Mayer et al., 1991); complement components such as C3, C4, factor B (Andoh et al., 1993); several receptors for cytokines including interleukin (IL)-2, IL-4, IL-6, interferon (IFN)-γ, and tumor growth factor (TGF)-β1 (Ullmann et al., 1992; Reinecker and Podolsky, 1995; Mulder et al., 1990; Raitano and Korc, 1993); eicosanoids (e.g. prostaglandin E_2 (PGE_2) in Laurent et al., 1998); and an array of pro-inflammatory cytokines and chemokines, i.e. tumor necrosis factor (TNF)-α and IL-8 (Eckmann et al., 1993a; Fierer et al., 1993; Jung et al., 1995; Dwinell et al., 1999). This "communication network" is finely regulated to induce or repress immune and inflammatory responses at mucosal surfaces. Such interaction involved the contact of epithelial cells to antigens causing the expression and production of cytokines. These affect other epithelial cells through an autocrine or paracrine effect and stimulate intestinal epithelial lymphocytes in the paracellular space and other immune cells such as neutrophils, mononuclear phagocytes, T cells, mast cells or eosinophils to release mediators that act on epithelial cells themselves as well as other cell population within the lamina propria (Maaser and Kagnoff, 2002).

Interactions occurring between bacteria and the different cell types of the immune system

The mucosal immune system needs to distinguish between "pathogenic" and "non-pathogenic" molecules. This immunological barrier is maintained by several mechanisms, including the degradation of potential immunogenic substances to low- or nonantigenic particles, and a constant communication between intestinal bacteria and different cell types of the immune system. The innate immune system exerts a permanent defence by stimulating the production of a mucus layer, by secreting antibacterial peptides and by maintaining the

integrity of the tight epithelium (Xavier and Podolsky, 2005). A breakdown of this controlled system results in bacterial invasion with subsequent intestinal infection or even in autoimmune pathogenesis such as CD or UC (Fiocchi, 1997a).

To avoid that soluble proteins and microbes do constantly cross the epithelium, the intestinal immune system used an elegant alternative to undergo an infection known as antigen presentation. This process involves the degradation of proteins to peptides that are associated with either major histocompatibility complex (MHC) class I or II molecules at the surface of antigen-presenting cells (APC). The latter express both MHC class I and class II molecules in addition to the ability to activate both CD8+ and CD4+ T cells.

The epithelium is interspersed in some regions by specialized cells, which do not present the brush border glycocalix on the apical cell surface. These cells, known as **M cells**, possess the microfolds instead of the brush border and have a huge invagination of the basolateral membrane, forming a pocket containing lymphocytes and/or macrophages. The lack of the brush border might facilitate the access and adherence of luminal antigens, while the basolateral membrane of M cells interacts with cells of the mucosal immune system. Found primarily in the Peyer's patches in the distal ileum of the small intestine, M cells use the transepithelial vesicular transport to carry antigens from the mucosal surface to specialized APCs in the underlying gut-associated lymphoid tissue (GALT) where they are presented to the immune system (Nagler-Anderson, 2001; Makala et al., 2004). M cells do not express MHC class II antigens and, therefore, they are unlikely to function as a professional APC (Beagley and Elson, 1992).

Another cell type capable of transporting antigens across the epithelium is the **dendritic cell** (DC). These cells are located in lymphoid as well as non-lymphoid tissues including sites of antigen entry. They are professional APC and use their dendrite-like process to sample antigens directly through epithelial tight junctions. During this process, the integrity of the epithelium is unaltered due to the rapid re-formation of the tight junctions by proteins expressed on both enterocytes and DCs (Nagler-Anderson, 2001). DCs process and complex antigens to MHC class II molecules which is then presented to T cells, eliciting an active immune response (Liu and MacPherson, 1993). This occurs due to the fact that DCs express molecules important to the T cell activation i.e. antigenic peptide, MHC class II and co-stimulatory molecules such as CD86, CD80 and CD40 (Sundquist et al., 2004). DCs exist in three stages of development: (1) precursor DC found in the blood and lymphatics, (2) tissue-residing immature DC, and (3) mature DC present within secondary lymphoid organs. In the absence of inflammatory stimuli or microbial products, DCs are in an immature state with high capacity to capture and process antigens and poor capacity to stimulated naïve T cells. However, after the contact with an antigen on the periphery or signals derived from

substances associated with infection or inflammation (e.g. lipopolysacharide (LPS), IL-1β, TNF-α), immature DC transform into mature DC with an exceptional capacity to stimulate T cells, particularly naïve T cells. This maturation step occurs on the way to lymphoid organs, when they are carrying encountered antigen (Makala et al., 2004). The migration of DCs from inflamed tissues to secondary lymphoid organs is mediated by chemokines. The dendritic cell-population can be divided in subgroups depending on the expression of other surface molecules, including CD8α, CD4, CD11b and CD205 (Sundquist et al., 2004).

Macrophages are also found in a large number in the GI tract and are the first phagocytic cells of the innate immune system that interact with microorganisms and their products that have penetrated the epithelium. They are predominantly located in the subepithelial lamina propria in the small and large intestine (Smythies et al., 2005). In addition, these cells are also present in the dome region of Peyer's patches (Hume et al., 1987). Intestinal macrophages have two important functions: (1) to protect the host against foreign pathogens and (2) to regulate mucosal responses to commensal bacteria. Human lamina propria macrophages express surface markers including MHC class II, CD13 (aminopeptidase N), CD68 and CD74 (invariant chain), and HLA-DR. However, intestinal macrophages do not express innate response receptors such as receptors for LPS (CD14), Immunoglobulin (Ig) A, IgG, CR3 and CR4, receptors for IL-2 and IL-3, and the integrin LFA-1 (Smythies et al., 2005). Resident tissue macrophages can be activated under the influence of various cytokines, which can modify their morphology, function, and metabolism. These changes reflect an increased capacity to enter tissues, phagocytose, and kill microorganisms and tumor cells in addition to an increased capacity for producing and secreting several mediators and cytokines including TNF-α, IL-1, IL-6 and IL-8. IFN-γ is considered to be the most important macrophage activator, inducing a vast array of phenotypic and functional changes not only on macrophages but also on other cells. It is produced by T cells, natural killer (NK) cells and probably by macrophages themselves (Boehm et al., 1997).

Intestinal **epithelial cells** are also able to function as APC. Some reasons for this is that these cells are poorly phagocytic and constitutively express or can be induced to express MHC class II molecules in addition to process and present antigens to primed T cells (Kagnoff and Eckmann, 1997). In a conventional antigen-presenting system, APC activate CD4+ helper T cells. In contrast, intestinal epithelial cells stimulate CD8+ suppressor T cells either by direct activation or by secretion of soluble factors, indicating that enterocytes play an important role in the down-regulation of mucosal immune responses (Toy and Mayer, 1996). In addition, epithelial cells produce a number of cytokines that affect immunoregulation and express cytokine receptors which can alter APC function (Madara and Stafford, 1989; Deem et al., 1991; Ciacci et al., 1993).

Spahn and Kucharzik presented an excellent overview of the lymphoid elements of the gut associated lymphatic system and their interaction in this cell communication network as shown in Figure 3.

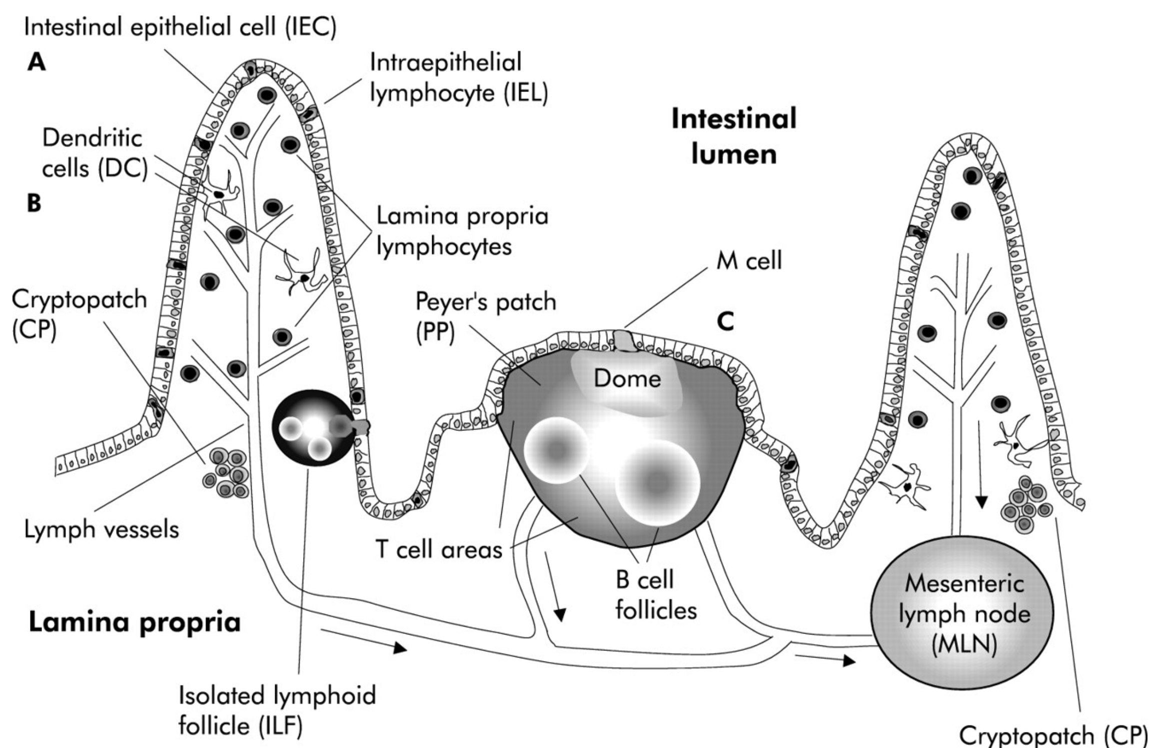


Figure 3: The lymphoid elements of the gut associated lymphatic system

There are three possibilities to take up luminal antigens: (A) through the intestinal epithelial cells (IEC), non-professional APC; (B) by lamina propria dendritic cells (DC); and by specialized epithelial cells (C) the M cells. Peyer's patches (PP) and mesenteric lymph nodes (MLN) represent the organized intestinal lymphoid follicles. The arrows demonstrate the lymph flow direction which goes from PP and villus lamina propria to the MLN (Spahn and Kucharzik, 2004).

Mucosal lymphocyte trafficking

Lymphocytes are distributed through the entire intestinal wall and are an important part of the mucosal immune system. The latter has three types of lymphocytes: (1) intraepithelial lymphocytes (IEL) which reside between mucosal epithelial cells, above the basement membrane; (2) lamina propria lymphocytes (LPL) and are located beneath the IEL layer, in the lamina propria between the epithelium and submucosa; and finally, (3) cells of the organized lymphoid tissue in the Peyer's patches (PP). This layer is found predominantly in the small intestine, appendix (solitary lymphoid nodules present in the colon and ileum) and MLN (James, 1991; Simecka, 1998). IEL and LPL are the largest single T cell sites in humans while PP lymphocytes are much less prominent in humans than in rodents.

IEL in humans are predominantly T lymphocytes and the majority have the CD8 phenotype, carrying either the $\alpha\beta$ or the $\gamma\delta$ T-cell receptor for antigens. Mouse and human small and large intestinal IEL comprise much higher percentages of $\gamma\delta$ T cells than are found in other lymphoid sites. Depending on the type of response needed, the composition of IEL is affected by their location along the intestine. An example for that is the difference in bacteria species found in the large compared to the small intestine. The former has a vast number of bacteria of many species present in its flora. $\gamma\delta$ T cells, which are abundant in the colon epithelium of the mucosa, participate in immune defence, immune regulation and tissue homeostasis (Ullrich et al., 1990; Makala et al., 2004). Moreover, intraepithelial $\gamma\delta$ T cells are involved in immune reactions in mucosal tissues by producing an array of pro-inflammatory cytokines and chemokines (Makala et al., 2004). Since a large number of IEL reside in the intestinal epithelium, a cross-talk between epithelial cells and IEL is essential for mucosal immune responses. The expression of IL-7 by enterocytes, for example, is important for the development of IEL (Nagler-Anderson, 2001) while intraepithelial $\gamma\delta$ T cells modulate growth and differentiation of epithelial cells (Boismenu and Havran, 1994).

LPL are predominantly T cells with both CD4+ (Beagley and Elson, 1992) and CD8+ (James, 1991) phenotypes, expressing $\alpha\beta$ T-cell receptor heterodimers (Fujihashi et al., 1994). These cells express activation markers, including the receptor for IL-2 (IL-2R+) - responsible for the proliferation, differentiation, and function of CD4+ cells (James et al., 1987) - and MHC class II (Zeitz et al., 1988). LP T cells are a specialized memory phenotype, implying that these cells already have been in contact with an antigen. Intestinal naïve T cells interact with antigens in organized lymphoid tissues (PPs and lymphoid follicles in the colon), differentiate, mature and enter the peripheral circulation. From the blood, activated lymphocytes home back to the LP as memory T cells (Jalkanen et al., 1989; Jalkanen, 1991). The activation of LP T cells is probably the result of the continuous antigenic and mitogenic challenge of the gut lumen. Intestinal LPLs have important functions in the gut: (1) CD4+ T cells help B-cell immunoglobulin (Ig) synthesis, mainly for the synthesis of IgA - the predominant type of Ig secreted by B lymphocytes of the gut – by producing cytokines such as IL-2, IL-4 and IFN-γ. (2) CD8+ T cells function as suppressors for Ig production (Lee et al., 1988). In addition to ligand-receptor interactions with other lymphocytes, LPL responses are also regulated by intestinal epithelial cells. The latter produce cytokines such as IL-8 and MIP-3α which directly affect polymorphonuclear cells and lymphocytes (Eckmann et al., 1993a; Izadpanah et al., 2001). These chemokines recruit neutrophils and lymphocytes, respectively, to the inflamed tissue.

Finally, lymphocytes are also found in PP, lymphoid aggregates that extend through the mucosa and submucosa of the small and large intestine. The surface covering the dome region of PP is the specialized follicle-associated epithelium, containing antigen-sampling M cells (already described above). The latter functions as “gateways to the immune system”, delivering antigens to APCs present in the PP subepithelial dome. The major sites for B cells are located in the B-cell follicles, a central region of the PP beneath the layer of M cells. This region is surrounded by DC. Primed T helper cells, migrating through this region, are able to interact with B cells associated with follicular DC, leading to Ig production. However, differentiation and maturation of IgA responses occur only after activated B cells leave the PP. Mature T cells are present in parafollicular regions of PP, in the periphery of the B cell follicles, and their activation is pivotal for the regulatory and effector activities of the immune system. T cells are also found near to the specialized epithelium where antigen presentation by macrophages and DC can occur. Moreover, CD4+ T helper cells and cytotoxic T lymphocytes are activated within PP and subsequently can migrate to other sites, such as the LP or intestinal epithelium (Kagnoff, 1993; Simecka, 1998; Nagler-Anderson, 2001; Makala et al., 2004).

Mucosal cytokine/chemokine production by intestinal epithelial cells

Inflammation is the most common type of response to microbial invasion or injury. The GI tract with its enormous mucosal surface is clearly more susceptible to such inflammatory responses. It is continuously exposed to a myriad of antigenic, mitogenic, mutagenic and toxic stimuli. The constant interaction of microorganisms with mucosal surfaces and the overlying epithelial cells can cause epithelial barrier disruption, and therefore, an acute inflammatory response. Inflammatory cells, including macrophages, neutrophils, and lymphocytes, are recruited into the inflammatory site.

Intestinal epithelial cells have the ability to present antigens to mucosal T cells in addition to produce cytokines in response to their environment. They produce cytokines that regulate the proliferation of intestinal LPL (Watanabe et al., 1995) and express functional cytokine receptors for several T cell-derived cytokines (Reinecker and Podolsky, 1995). This reciprocal exchange of regulatory signals is altered during intestinal inflammation, where epithelial cells express or up-regulate the expression of several functional cytokines and cell adhesion molecules (Huang et al., 1996). They also secrete a variety of pro-inflammatory cytokines affecting the leukocyte activity (Jung et al., 1995).

Recent *in vivo* studies have provided a number of evidences that cytokines and chemokines are crucial mediators of inflammatory responses and tissue injury in intestinal inflammation (Ajuebor and Swain, 2002). Intestinal epithelial cells produce a range of

pro-inflammatory cytokines, including IL-1α, IL-1β, IL-6 and TNF-α, and chemokines. These are a large family of small molecules (7-15kDa) that participate in immune and inflammatory responses by chemoattracting and activating polymorphonuclear leukocytes (PBL) to the injured tissue (Baggiolini et al., 1994; Baggiolini, 1998; Luster, 1998). Based on the arrangement of their N-terminal cysteine residues, chemokines are subdivided into four classes: C, CC, CXC, and CX3C. Their biological action are mediated through 7-transmembrane G protein-coupled receptors present on the surface of target cells (Balkwill, 1998; Luster, 1998; Murphy et al., 2000). IL-8, growth regulated oncogene (GRO)-α, GRO-γ, macrophage inflammatory protein (MIP)-2 and IFN-inducible protein (IP)-10 belong to the CXC chemokine subfamily and recruit mainly neutrophils to the inflamed tissue (Cuenca et al., 1992; Eckmann et al., 1993a; Eckmann et al., 1993b; Izadpanah et al., 2001; Kwon et al., 2002). MIP-3α, a member of the CC chemokines, recruits monocytes/macrophages and subpopulations of T cells (Yang et al., 1997; Dwinell et al., 1999; Ajuebor and Swain, 2002). Regulated on activation normal T cell expressed and secreted (RANTES), a chemoattractant for T cells and eosinophils, is also expressed by intestinal epithelial cells (Yang et al., 1997). The secretion of these chemokines by activated epithelial cells suggest that - in addition to orchestrating the initiation of mucosal inflammatory and immune responses - these cells have an important function in initiating the mucosal influx of inflammatory cells (Kagnoff and Eckmann, 1997; Dwinell et al., 1999).

Epithelial cells produce IL-1 mRNA; however, the intracellular protein levels are very low. It was suggested that IL-1 would act on adjacent cells to induce or amplify an inflammatory response or induce wound healing (Eckmann et al., 1995). TNF-α is also produced by epithelial cells in response to bacteria invasion or an extern stimuli (Keshav et al., 1990; Tan et al., 1993), leading to increased production and activation of other pro-inflammatory cytokines (e.g. IL-8, MIP-3α and TNF-α itself) and factors that promote intestinal inflammation (Neurath et al., 1997; Van Deventer, 1997). It also acts on the intestinal epithelium altering the epithelial barrier function by disrupting the intercellular tight junctions of the enterocytes (Ma et al., 2004). Furthermore, intestinal epithelial cells have the potential to produce the pro-inflammatory cytokine IL-6 indicating its importance in mucosal inflammatory responses (Akira et al., 1993). There are no data related to the expression of IL-2, IL-4, IL-5, IL-12, or IFN-γ by intestinal epithelial cells. This suggests that cytokines secreted by these cells are more involved in initiating and regulating the innate mucosal inflammatory responses rather than antigen-specific mucosal immune responses (Kagnoff and Eckmann, 1997). Epithelial cells also express receptors for several cytokines (e.g. IFN-γ, IL-1, TNF-α, TGF-β1; IL-2, IL-4, IL-7 and IL-9), which is an indicative that they respond to a range of signals from the underlying mucosa (Reinecker and Podolsky, 1995).

Enterocytes express mediators involved in inflammatory responses

Intestinal epithelial cells produce several mediators involved in inflammation. One of them is the COX-2-dependent prostaglandin E_2 (PGE_2), which is increased in intestinal inflammation. After bacteria invasion or extern stimuli, TNF-α stimulates the expression of COX-2, the key enzyme for PGE_2 formation. The latter can directly induce epithelial cells to increase chloride secretion (Eckmann et al., 1997) and indirectly stimulate enteric nerves to release neurotransmitters that activate epithelial ion transport (Eberhart and Dubois, 1995). COX-2 was shown to be also expressed in surface epithelial cells in inflamed areas of CD and UC (Singer et al., 1998).

During chemokine-dependent recruitment of inflammatory cells into the mucosa, intestinal epithelial cells can interact with neutrophils and lymphocytes by expressing ICAM-1. Its ligand β2 integrin is expressed on neutrophils and lymphocytes. ICAM-1 is up-regulated on epithelial cells after bacterial invasion or stimulation with pro-inflammatory cytokines, including IFN-γ, IL-1β and TNF-α (Kelly et al., 1994). Thus, lymphocyte-enterocyte interactions are enhanced in intestinal inflammation (Brynskov et al., 1992). The function of ICAM-1 on the apical surface of intestinal epithelial cells is to maintain neutrophils that have closely transmigrated through the epithelium (Huang et al., 1996).

Another mediator found in intestinal epithelial cells during the course of intestinal inflammation is nitric oxide (NO). It is produced by the inducible NO synthase (NOS2) and affects multiple gastrointestinal functions, including blood flow and mucosal inflammation (Kagnoff and Eckmann, 1997).

Inflammatory bowel disease (IBD)

IBD includes two intestinal inflammatory disorders: Crohn's disease (CD) and ulcerative colitis (UC). In CD, the intestinal area affected comprises commonly the lower ileum; however, the disease can flare up anywhere, including the colon. Interestingly, "patches" of normal tissue can be found between affected areas. In contrast, UC is characterized by contiguous inflammation of the colonic lamina propria without "patches" of normal tissue between injured areas. Both CD and UC are dependent on genetic and environmental factors (Podolsky, 1991) and are characterized by prominent ulcerative lesions and a huge leukocyte infiltration in the bowel wall. CD and UC are associated with a Th1 and Th2 immune responses, respectively. In CD a huge amount of T cells infiltrate the intestinal mucosa, while in UC neutrophilic infiltration prevails (Fiocchi, 1998). Table 1 resumes some features of both CD and UC.

Table 1. Features of CD and UC

Component	CD	UC
Environmental factors	Detrimental effect of smoking	Beneficial effect of smoking
	Symptoms improved by selected diets	No beneficial effect of diet
	Increased intestinal permeability in healthy relatives	Normal intestinal permeability in healthy relatives
Genetic associations	Largely different from UC	Largely different from CD
Microbial agents	Important role of bacterial flora	Limited role of bacterial flora
	Some association with measles virus and M. paratuberculosis	No association with measles virus and M. paratuberculosis
Humoral immunity	Moderate antibody secretion	Prominent antibody secretion
	Limited evidence for autoimmunity	Evidence for autoimmunity
	Weak association with antineutrophil cytoplasmic antibodies	Strong association with antineutrophil cytoplasmic antibodies
Cell-mediated immunity	Prominent T-cell infiltration in the mucosa	Prominent neutrophil infiltration in the mucosa

	Hyperreactive T cells Resistance of T cells to apoptosis (?)	Normal/hyporeactive T cells Normal T-cell apoptosis (?)
Cytokines and mediators	Moderate production of eicosanoids Th1-like profile Increased cytokine production in involved and uninvolved mucosa	Prominent production of eicosanoids Th2-like profile Increased cytokine production limited to involved mucosa

Table was adapted from (Fiocchi, 1998).

Many cytokines and chemokines are markedly increased in intestinal tissues from patients with CD and UC. Expression levels of IL-1β, IL-6, TNF-α, and IFN-γ in the colonic tissue from patients with IBD was shown to be elevated (Rogler and Andus, 1998). In addition, this increase was shown to play an important role in sustaining inflammatory responses in animal models (Elson et al., 1995; Strober et al., 2002). IL-8 levels were increased in rectal biopsies from patients with active CD or UC (Mahida et al., 1992; Izzo et al., 1993; Ajuebor and Swain, 2002). Subsequently, the expression of this chemokine was correlated with the severity of inflammation found in IBD (Mazzucchelli et al., 1994). During the active phase of IBD, IL-8 expression is also increased in neutrophils and macrophages in addition to intestinal epithelial cells (Ajuebor and Swain, 2002). Consistent with these findings is the increased expression of CXCR1, the receptor for IL-8, on macrophages and neutrophils of patients with active UC (Williams et al., 2000). This suggests that the role of IL-8 during chronic colitis is to attract CXCR1-bearing inflammatory cells to colonic tissue, where activation of these cells leads to tissue ulceration.

Plant extracts affect GI inflammation

Many dietary plants and their phytochemicals possess anti-inflammatory properties. Among them, *Boswellia serrata* and its boswellic acids, EGCG from green tea, and curcumin from turmeric have been shown to inhibit inflammatory processes in a variety of models, as described in the following chapters of this thesis. Other natural plants have also been shown to affect intestinal inflammation, including *Allium sativum* (garlic), *Plantago ovata* seeds, *Polygalae* root, Ispaghula husk, *Polygonum tinctorium*, and germinated barley foodstuff. Garlic extract ameliorated inflammation associated with IBD by inhibiting Th1 and inflammatory cytokine production (e.g. IL-12, IFN-γ, TNF-α, and IL-8) and up-regulating the synthesis of the anti-inflammatory cytokine IL-10 (Hodge et al., 2002). In another study, seeds of *Plantago ovata* were shown to be efficient in maintaining remission in UC. Furthermore, their effect was comparable to mesalamine, a drug used in the treatment of colitis. It was demonstrated that colonic fermentation of the *Plantago ovata* seeds yields butyrate, a short-chain fatty acid (SCFA) which serves as nutrient for intestinal epithelial cells. The presence of butyrate in IBD reduced inflammation by inhibiting the production of cytokines and the activation of the transcription factor nuclear factor – *kappa* B (NF-κB) (Fernandez-Banares et al., 1999). In addition, butyrate protects the intestinal mucosa against injury and promotes mucosal healing (Scheppach et al., 1992). Moreover, husk of the *Plantago ovata* seeds, also known as Ispaghula husk, was shown to efficiently alleviate GI symptoms in quiescent UC in humans (Hallert et al., 1991). In an animal model of colitis, *Polygalae senega* root reduced the degree of inflammation by regulating the associated-cytokine production of IEL (mainly IFN-γ and IL-4) and diminishing PBL infiltration (Hong et al., 2002b). Prebiotic products have also been studied in IBD. Germinated barley foodstuff (GBF), which contains glutamine-rich protein and hemicellulose-rich fiber, has shown to prevent colitis activity - by reducing epithelial inflammatory responses - and body weight loss in an animal model of colitis. Administration of GBF augments SCFA production which increases cecal butyrate levels, improving colonic epithelial cell proliferation (Kanauchi et al., 2003). Furthermore, the flavonoid Bacalein, isolated from the roots of *Scutellaria baicalensis* Georgi (Labiatae), reduced and ameliorated the inflammatory symptoms of the dextran sulfate sodium (DSS)-induced colitis. Its effect was reported to be similar to that of sulfasalazine, the reference drug used for the treatment of ulcerative colitis in humans (Hong et al., 2002a). Also the phytochemical tryptanthrin, isolated from the indigo plant *Polygonum tinctorium*, may have therapeutic effect on colitis (Micallef et al., 2002).

OBJECTIVES

The present thesis was undertaken to identify natural anti-inflammatory compounds with the ability to prevent and/or treat intestinal inflammatory diseases, including IBD. However, the purpose of this work was not to search for a new substance with similar but lower side effects to the commercial drugs already “in use” to treat such disorders. This project focused more on the effects of natural compounds and whole plant extracts in reducing inflammatory features by regulating, for example, unbalanced cytokines/chemokines produced in response to an antigen or pathogen. Such a compound or even the whole plant extract could improve life-style by affecting inflammation in the beginning of the “inflammatory” cascade, reducing the acute phase and preventing or delaying the relapse of the disease. Furthermore, natural substances could be use in IBD where the usage (application rate and frequency) of highly efficient anti-inflammatory drugs could be reduced.

Specific goals were defined as follows:

- Development and characterization of an appropriate human *in vitro* cell culture model that is closely related to the human intestinal epithelium, where GI inflammation can be mimicked.
- Characterization of the anti-inflammatory properties of isoflavones and polyphenols on mediators involved in GI inflammation.
- Determination of the anti-inflammatory effects of natural extracts on GI function.
- Identification of the cellular and molecular mode of action of related compounds in intestinal inflammatory responses through a comprehensive gene expression analysis.

MATERIAL AND METHODS

Reagents

Recombinant human TNF-α and IL-1β were purchased from Peprotech (London, UK). Recombinant human IL-8, purified mouse anti-human IL-8 and biotinylated mouse anti-human IL-8 were obtained from BD Pharmingen (San Diego, CA, USA). Recombinant human MIP-3α/CCL20, anti-human MIP-3α/CCL20 and biotinylated anti-human MIP-3α/CCL20 were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). Cell culture reagents were obtained from GIBCO (Basel, Switzerland). EGCG (Teavigo™), curcumin and resveratrol were obtained from DSM Nutritional Products (Basel, Switzerland). Extracts of BS and RO were obtained from Sabinsa Corporation (Piscataway, NJ, USA). IκBα C-21 (sc-371), NF-κB p65 (sc-109) were purchased from Santa Cruz Biotechnology (Labforce, Nunningen, Switzerland). Anti-actin (rabbit; A-2066) antibody was obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Primers and probes were purchased from Sigma-Genosys (Homefield Road, Haverhill, UK).

Cell culture

HT29 and T84 cells were obtained from ATCC (LGC Promochem, Molsheim, France). HT29 and T84 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, 50 μg/mL streptomycin, L-glutamine and nonessential amino acids (NEAA, Invitrogen, Basel, Switzerland). Cells were maintained in a water-saturated atmosphere of 95% O_2 and 5% CO_2 at 37°C. Cells were used between passages 20 to 50. For experiments, cells were seeded at 0.5×10^6 and 1×10^6 cells/well into 12-well-plates and at 0.2×10^5 cells/well into 96-well-plates and used after 3 or 4 days of pre-culture. They were starved in DMEM containing 0.25% FBS for 18 hours before the experiments. Cells were stimulated with TNF-α (100 ng/mL) and/or IL-1β (5 ng/mL) in phenol-free DMEM containing 0.25% FBS. EGCG, extracts of BS containing a minimum of 40% β-BAs, and of RO which contain 15% carnosic acid and 20% ursolic acid were dissolved in DMSO and added to the culture medium concomitantly with the stimulus. Where appropriate, DMSO was added to the cell culture at a final concentration of 0.5% (v/v).

Cytotoxicity

Released lactate dehydrogenase (LDH) was measured in culture supernatants immediately after harvesting, using LDH enzyme controls as standard (Sigma, St. Louis, USA). In a microtiter plate, 20 μL of undiluted culture supernatants or standard were mixed with β-NAD

solution (172 mM) and Tris acetate buffer (13.6 g/L Tris base, 12.8 g/L KCl, 5.08 g/L L-lactate, 1 g/L NaN_2, pH 9.3). The kinetics of the reaction was measured at 340 nm at 30°C for 5 min. To determine the total LDH contents, cells were lysed in the presence of a buffer containing 0.1 M NaCl, 1 mM EDTA, 10 mM Tris/HCl and 1% Triton X-100, and protease inhibitors. This protocol was adapted from Korzeniewski and coworker (Korzeniewski and Callewaert, 1983).

Enzyme-linked immunosorbent assay (ELISA) and Enzyme Immunoassay (EIA)

PGE_2 assay. The amount of PGE_2 in culture supernatants was assayed with EIA kits (obtained from Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's instructions.

IL-8 assay. Nunc Maxisorp 96-well microtitre plate (Fisher Scientific, Wohlen, Switzerland) was coated with 3 μg/mL goat anti-human IL-8 antibody (Pharmingen, Becton Dickinson, Heidelberg, Germany) in 50 μL binding buffer (0.1 M Na_2HPO_4, pH 9.0) overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBS-T), wells were blocked with PBS containing 10% FBS and incubated for two hours at room temperature. After further washing with PBS-T, 100 μL aliquots of culture supernatant or recombinant IL-8 were added and incubated overnight at 4°C. To detect bound IL-8, 0.25 μg/mL biotinylated anti-human IL-8 antibody (Pharmingen, Becton Dickinson, Heidelberg, Germany), mixed in blocking buffer, was added for one hour at room temperature. Streptavidin and alkaline phosphatase (DAKO, Glostrup, Denmark), diluted 1:50 in PBS, were pre-incubated for 30 min at room temperature to form conjugates. These conjugates were then added to the plate and incubated at 37°C for 1 hour. After washing, 50 μL of p-nitrophenyl phosphate substrate (1 mg/mL) (Sigma, Steinheim, Germany) was added to each well and incubated at 37°C for 20 min. The optical density at 405 nm was read using a microtitre plate photometer (Molecular Devices, USA). All determinations were performed in triplicates.

MIP-3α assay. Nunc immunosorb microtitre plate (Fisher Scientific, Wohlen, Switzerland) was coated overnight at 4°C with 2 μg/mL goat anti-human MIP-3α / CCL20 antibody (R&D Systems, Wiesbaden–Nordenstadt, Germany) diluted in 50 μL of carbonate coating buffer (pH 9.6). After washing with PBS-T, wells were blocked with PBS, containing 1% BSA, 5% sucrose and 0.05% NaN_3, for one hour at room temperature. After further washing with PBS-T, 50 μL aliquots of culture supernatants or recombinant MIP-3α were added for two hours at room temperature. To detect bound MIP-3α, 0.5 μg/mL biotinylated anti-human MIP-3α antibody (R&D Systems, Wiesbaden – Nordenstadt, Germany), mixed in blocking buffer, was added and the plate was incubated for one hour at room temperature. Streptavidin-biotin alkaline phosphatase complexes (DAKO, Glostrup, Denmark) were formed

and immune complexes visualized and measured as described above. All determinations were performed in triplicates.

Whole cell protein extracts

RAW 264.7 cells were stimulated with or without LPS (1 μg/mL) for 20 min. Cells were harvest and lysed in NETT-C (0.1 M NaCl; 10 mM Tris/HCl, pH 7.6; 1 mM EDTA; 1% Triton X-100 mixed with a cocktail of protease inhibitors (Complete™, Roche Diagnostics, Mannheim, Germany)). Lysates were kept on ice for 15 min, sonicated (Branson Sonifier, 30% duty cycle, output control 3-4), cleaned by centrifugation (14000 rpm for 2 min at 4°C) and stored at -80°C until use. Protein concentration of extracts was measured using bicinchoninic acid (BCA) reagents according to manufacturer's instruction (Pierce, Rockford, IL, USA).

Cytoplasmic and Nuclear protein extracts

Cells were harvested after 10 and 20 min of incubation, kept on ice and washed twice with ice-cold PBS. After scraping them off with 400 μL of ice-cold buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; completed with protease inhibitors cocktail (Complete™)). Twenty five μL of 10% NP-40 (Fluka, Steinheim, Germany) were added to the cells, mixed by vortexing for 10 sec and centrifuged at 14000 rpm, 4°C for 15 sec. Cytoplasmic proteins (supernatant) were collected in a new tube. The pellet was resuspended in 50 μL of ice-cold buffer C (20 mM HEPES, pH 7.9; 0.4 M NaCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; completed with protease inhibitors at a final volume of 10 mL), mixed and incubated for 15 min at 4°C. After centrifugation (14000 rpm, 4°C for 5 min), nuclear extract was obtained by transferring the supernatant to a new tube. Both cytoplasmic and nuclear extracts were frozen in liquid nitrogen and stored at -80°C. Protein concentration of extracts was measured as described above.

Immunoblot

Protein samples were mixed with one volume Tricine-SDS sample buffer (Invitrogen, Carlsbad, CA, USA) containing 10% of 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and heated at 95°C for 5 min. Ten to thirty μg of proteins were separated by electrophoresis in 10-20% of Tricine gels (Novex, San Diego, CA, USA) and transferred to a nitrocellulose membrane. Membranes were incubated for 2 hours in blocking reagent (BR; Roche Diagnostics, Mannheim, Germany). Antibodies against IκB-α, NF-κB p65 or actin were diluted with PBS-T (PBS, 0.05% Tween 20) / 0.2 x BR (final dilution 1:1000). After washing with PBS-T, membranes were incubated with antibodies for 2 hours or overnight. Appropriate secondary antibody conjugated to horseradish peroxydase (*i.e.* donkey anti-rabbit IgG) was

first diluted 1:10 000 in PBS-T and incubated with the membrane for 45 min. Immune complexes were visualized by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ, USA) using high performance chemiluminescence film (Amersham).

Analysis of NF-κB DNA binding using Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotide probe labeling. 1 μL double stranded oligonucleotide (25 ng/μL; 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 3'-TCA ACT CCC CTG AAA GGG TCC G-5'; Promega, USA) was radioactively labeled using 5 μL 10x kinase buffer (provided with the kinase), 1 μL T4-polynucleotide kinase (New England Biolabs) and 37 μL sterile double distilled H_2O. By using 5 μL [γ- ^{33}P]-ATP (3000 Ci/mmol; Amersham, Freiburg, Germany) as a substrate, the radioactive γ-phosphate of [γ- ^{33}P]-ATP is transferred to the 5'-OH in the DNA, generating a 5'-[^{33}P]-phosphate-labeled oligonucleotide. The mixture was incubated for 60-90 min at 37°C and purified by using MicroSpin G-25 columns (Amersham, Freiburg, Germany).

EMSA. Ten to 20 μg of protein extracts (nuclear and cytoplasmic) were added to a reaction mixture containing 20 μg of bovine serum albumin (Sigma, Deisenhofen, Germany), 2 μg of poly-desoxyinosinyl-desoxycytidylic acid, double stranded (poly(dIdC); Roche Molecular Biochemicals, Mannheim, Germany), 2 μL of buffer D+ (20 mM Hepes, pH 7.9; 20% glycerol; 100 mM KCl; 0.5 mM EDTA; 0.25% NP-40; 2 mM DTT; 0.1% phenylmethylsulfonyl fluoride), 4 μL of Buffer F (20% Ficoll 400, 100 mM Hepes, 300 mM KCl, 10 mM DTT, 0.1% phenylmethylsulfonyl fluoride), and 100000 cpm (Cerenkov) of a [^{33}P]-labeled oligonucleotide, made up to a final volume of 20 μL with distilled water. Samples were incubated at room temperature for 25 min. After the incubation, samples were loaded on a non-denaturating 4% polyacrylamide gel and electrophoresis was run at 200V (22 to 14 mA) for 90 min. The gel was transferred to a Whatman 3MM paper (Schleicher & Schüll, Kassel, Germany) and dried under vacuum. Subsequently, the gel was exposed to a Phosphoimager BAS film (Fujifilm) for 24 hours. Detection was performed by a Phosphoimager (Fujifilm).

RNA isolation and reverse transcription (RT)

Total RNA was isolated using the RNeasy® Mini Kit from Qiagen (Hilden, Germany). Extraction was performed according to the manufacturer's directions. Matrix-bound RNA was eluted with 30 μL of RNase-free water. The quantity and purity of RNA was determined by measuring the optical density at 260 nm and 280 nm. Subsequently, 1.5 to 3.5 μg of total RNA were converted to first strand cDNA using SuperScript IITM reverse transcriptase (Invitrogen, Basel, Switzerland) and 50 ng/μL random hexamers (Microsynth, Balgach, Switzerland). The

conditions for the cDNA synthesis were: 5 min at 70°C for random primer annealing followed by cooling on ice; 10 min at room temperature, 50 min at 42°C and 15 min at 70°C for the denaturation step; and 20 min at 37°C for the RNase H digestion. The cDNA was subsequently diluted to 100 μL in water and stored at –80°C.

Quantitative Real Time RT-PCR analysis

The analysis of mRNA expression profiles was performed with multiplex quantitative RT-polymerase chain reaction (PCR). In a 50 μL PCR reaction, 3 μL cDNA (corresponding to 30-50 ng of total RNA input) were amplified in an ABI Prism 7700 Sequence Detector (PE Biosystems, Foster City, CA, USA), using 2x Taqman Universal PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland), 50 nM primers and 100 nM probe for the 18S rRNA reference gene, and 300 nM primers and 100 nM probe for genes of interest. The probe for 18S rRNA was fluorescently labelled with VIC^{TM} on the 5' end and TAMRA on the 3' end (Applied Biosystems, Rotkreuz, Switzerland), whereas probes for the genes of interest were labelled with 6-carboxy-fluorescein (FAM) on the 5' end and TAMRA on the 3' end. The PCR amplification conditions consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 and 60 sec at 95°C and 60°C, respectively. For gene sequences see Table 2.

Table 2. Gene sequences used in quantitative RT-PCR.

Gene	Forward and reverse primers (5'→ 3')	Probe (5' → 3')
18S rRNA	CGGCTACCACATCCAAGG CGGGTCGGGAGTGGGT	TTGCGCGCCTGCTGCCT
IL-8	ACTGACATCTAAGTTCTTTAGCACTCC GCCTTCCTGATTTCTGCAGC	TGGCAAAACTGCACCTTCACACAG
TNF-α	TCGAACCCCGACTGACAA AGCTGCCCCTCAGCTTG	CCTGTAGCCCATGTTGTAGCAAACCC
COX-2	GCCCTTCCTCCTGTGCC AATCAGGAAGCTGCTTTTTACCTTT	ATGATTGCCCGACTCCCTTGGGTGT
MIP-2	AGTCCCCCGGACCCC GCCCATTCTTGAGTGTGGC	CTGCGCCCAAACCGAAGTCAT
MIP-3α	TGTCAGTGCTGCTACTCCACCT CCAAGACAGCAGTCAAAGTTGC	TGCGGCGAATCAGAAGCAGCA
GRO-α	AAGTCCCCCGGACCCC TTCCGCCCATTCTTGAGTGT	CTGCGCCCAAACCGAAGTCATAGC
GRO-γ	GTCCCCCGGACCCCA ACAAGCTTTCTTCCCATTCTTGAG	CGCCCAAACCGAAGTCATAGCCAC
IL-1α	CTGGAGGCCATCGCCA TGCTCAGGAAGCTAAAAGGTGC	TGACTCAGAGGAAGAAATCATCAAGCCTAGGTC
IL-1β	GGCCTCAAGGAAAAGAATCTGTAC GGGATCTACACTCTCCAGCTGTAGA	TCCTGCGTGTTGAAAGATGATAAGCCCA
PGES	TGTACGTGGTGGCCATCATC CTCAGGGCATCCTCGGG	AGGCTGCGGAAGAAGGCCTTTGC
MMP-14	CTGCCGAGCCTTGGACTG CGTGCACAGCCACCAGG	CAGGAATGAGGATCTGAATGGAAATGACATCT
5-LOX	CGGCGATGTCGAGGTTG TGTGAATTTGGTCATCTCGGG	TGAGGGATGGACGCGCAAAGTTG
RANK	GCTTGCTGCATAAAGTTTGTGA TGTTGCCGGCGACCA	AGGCAAGGCCCTGGTGGCC
IEX-1L	TCTCTACCCTCGAGTGGTGAGTATC ACTCCAGGGCAGCGCA	CCGAAGTGGGCATTCGCGGT
β-integrin 5	CCCAAAATGTGCCTGGTG CCGAGAGGTGATGGACCG	TCCAAAGAGGACTTCGGAAGCCC
IP-10	TGAAATTATTCCTGCAAGCCAA CAGACATCTCTTCTCACCCTTCTTT	GTCCACGTGTTGAGATCATTGCTACAATG
RANTES	ACCAGTGGCAAGTGCTCCA GCACACACTTGGCGGTTCTT	CCCAGCAGTCGTCTTTGTCACCCG
FRA-1	CACGACCCATCTGCAAAA GCCACTGGTACTGCCTGTGTC	CCCGGAAGGAGCCAAGGAGGG
CFb	TGCTGACCCCAATACTTGCA GAATGAAACGACTTCTCTTGTGAACT	AGGTGATTCTGGCGGCCCCTTG

Primers and probes sets were designed with the Primer Express^{TM} software (Applied Biosystems, Rotkreuz, Switzerland).

mRNA abundance was defined using the ΔC_T method according to the manufacturer's protocol (ABI Prism 7700 Sequence Detection System, Rotkreuz, Switzerland). Briefly, the ΔC_T for the gene of interest was determined as the difference between the C_T values for the gene of interest and 18S rRNA, where the C_T value is the cycle threshold. The standard deviation (SD) was obtained from ΔC_T of cDNA samples assayed in duplicate, where the upper and lower errors were defined as $2^{-(\Delta C_T - SD)}$ and $2^{-(\Delta C_T + SD)}$, respectively. $\Delta\Delta C_T$ was determined as the difference in ΔC_T of unstimulated cells compared to that of each treatment group. The mRNA level for the gene of interest was determined as $2^{-\Delta\Delta C_T}$ and, therefore, reflects changes relative to unstimulated cells. Each cDNA sample was assessed in duplicates.

Affymetrix GeneChip® hybridization

GeneChip® hybridization was carried out as described previously (Siler et al., 2004). Briefly, RNA (10 μg) was subjected to double-stranded (ds)-cDNA synthesis with T7-d$(T)_{24}$ primers and the Superscript™ II choice system (Invitrogen), followed by an *in vitro* transcription reaction (T7 MegaScript kit, Ambion, Austin, TX) to generate the hybridization probes. The cRNA was labeled by incorporation of biotin-11-CTP and biotin-16-UTP (Roche Molecular Systems, Penzberg, Germany). cRNA (10 μg) was fragmented by incubation in 40 mM Tris-acetate, pH 8.1; 100 mM KOAc; and 30 mM MgOAc for 35 min at 95°C. Samples were hybridized to the human genome U133A array (Affynetrix, Santa Clara, CA) for the screening of transcripts as described in the Gene Chip Expression Analysis Technical Manual (Affymetrix, Oxford, UK). Each treatment was done in quadruplicates and was represented by four chips. Fragmented cRNA was hybridized overnight with 1x MES and 0.01% Tween-20 at 45°C. The arrays were washed at a maximal stringency of 1x MES and 0.01% Tween-20 at 50°C (Fluidics program EukGE-WS2). Hybridization signals were detected by streptavidin-phycoerythrine staining (Molecular Probes, Leiden, Netherlands), which was amplified by incubation with anti-streptavidin antibody (Vector Labs, Orton Southgate, Great Britain) and staining with streptavidin-phycoerythrine. Subsequently, the microarrays were subjected to laser scanning (GeneChip® Scanner 3000; Affymetrix, Bedford, MA, USA), and the hybridization signals were analyzed with the GeneChip Analysis Suite MAS 5.0 (Affymetrix).

GeneChip® data analysis

The gene chip data analysis was carried out using RACE-A (Roche Affymetrix Chip Experiment-Analysis), a Roche proprietary software package for differential expression analysis as described (Siler et al., 2004). Briefly, the workflow supported by RACE-A comprises the following steps: (1) selection and quality assessment of probe arrays; (2) reading probe set intensities reported by Affimetrix chip analysis software; (3) assignment of

chip replicates to experimental conditions; (4) comparisons of experimental conditions; (5) filtering of comparison results based on a variety of attributes calculated by RACE-A; and (6) output to Excel® table format.

During step (3), probe arrays were normalized against the mean signal intensity of each chip and outlier genes were removed. Gene expression in each treatment was compared with that of unstimulated cells. Treatment-induced changes in gene expression levels are reported as “change factor” relative to unstimulated cells. Change factors > 0.5 represent an up-regulation by EGCG compared to control, and < 0.5 indicate a down-regulation. Changes were considered significant, if either the *P* value was < 0.01 for a single gene regulated by EGCG, or if a group of genes involved in a certain pathway or biological process was consistently regulated in the same direction.

Statistical analysis

The upper and lower limits of mRNA expression levels were calculated as described above. All other values are presented as mean ± SD and all statistics were evaluated double-sided using a two-sample equal variance Student’s *t*-test, assuming independent variance. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were considered to be significant. For the microarray analysis, only genes with $p < 0.01$ were used.

Chapter 1

EGCG Impairs Chemokine Production in Human Colon Epithelial Cell Lines

J Pharmacol Exp Ther. **315(3)**:1172-80, 2005

ABSTRACT

A major component in green tea, epigallocatechin-3-gallate (EGCG), is reported to interfere with different steps of a number of inflammatory pathways. After oral administration, EGCG is retained in the gastro-intestinal tract, where it is thought to exert preventive functions against inflammatory bowel disease (IBD) and colon cancer. In this study, the human colon adenocarcinoma cell lines HT29 and T84 were used to investigate the effect of EGCG on intestinal inflammation. HT29 and T84 cells were stimulated with tumor necrosis factor (TNF)-α to induce the "inflammatory" condition and to trigger the inflammatory cascade *in vitro*, and treated with EGCG to study its effect on inflammatory processes. The secretion of the chemokines interleukin (IL)-8 and macrophage inflammatory protein (MIP)-3α and prostaglandin E_2 (PGE_2) was determined by ELISA. The gene expression level was measured by quantitative real-time (RT)-PCR. Treatment of TNF-α-stimulated HT29 cells with EGCG dose-dependently inhibited the synthesis of IL-8, MIP-3α and PGE_2. Treatment with EGCG also inhibited the production of IL-8 and MIP-3α in TNF-α-stimulated T84 cells. Gene expression analysis in both HT29 and T84 cells revealed that EGCG down-regulates genes involved in inflammatory pathways. This study shows that EGCG acts at different levels on the production of chemokines and PGE_2 in the chemokine and eicosanoid-pathways of colon epithelial cells. Therefore, EGCG might prove useful for the prevention and/or attenuation of colonic disorders.

INTRODUCTION

The constant exposure of the intestinal epithelium to diverse types of nutrients and microorganisms present in the natural flora leads to a permanent “stress state” for the enterocytes. Mucosal surfaces of the intestinal tract form one of the main routes for microbial pathogens to enter a host, and are important sites of microbially-induced diseases. In some food allergies or after invasion of the epithelium by pathogenic bacteria, the intestinal mucosa is irritated. This might lead to acute inflammation, characterized by an excessive production of inflammatory mediators. These are, in part, responsible for the recruitment of specific cell types, for example, macrophages/monocytes, lymphocytes or neutrophils (Kagnoff, 1996).

During intestinal inflammation, polymorphonuclear leukocytes (PML), including neutrophils, migrate into the mucosa. In the intestine, an exacerbation of destructive processes occurs due to different pro-inflammatory and chemoattractant molecules. The latter are chemokines, a large family of small proteins, which are involved in innate immune and inflammatory responses by chemoattracting to, and activating leukocytes at the site of inflammation, and up-regulating adhesion molecules important for leukocyte trafficking (Baggiolini and Moser, 1997; Dwinell et al., 1999). Biological actions of chemokines are mediated through G-protein-coupled receptors which are present on the surface of target cells (Balkwill, 1998; Murphy et al., 2000). These receptors exhibit overlapping specificity for chemokines within each subfamily, and the cellular expression of respective receptors determines which cell types respond to a given chemokine. Based on the arrangement of their N-terminal cysteine residues, chemokines are subdivided into four subfamilies, including CXC and CC. Cells responsive to chemokines recognize concentration gradients and migrate towards the source of chemokine secretion. IL-8, MIP-2 and growth-regulated oncogene (GRO)-α and GRO-γ are CXC chemokines which attract and activate neutrophils, whereas the CC chemokines, such as MIP-3α, activate leukocyte populations, including monocytes, T lymphocytes, DC and, to a lesser extent, neutrophils (Ajuebor and Swain, 2002). Chemokines contribute to the perpetuation of inflammatory processes, increasing chronic intestinal inflammation and mucosal destruction. Secreted chemokines and their specific chemokine cell-surface receptors play a crucial role in the final composition of leukocytes present in the inflamed intestine (MacDermott, 1999; Banks et al., 2003).

Prostaglandins are also involved in the regulation of a variety of physiological and pathological processes in the immune response and in inflammation. Prostaglandin E_2 (PGE_2) and other prostanoids are generated through two bifunctional enzymes, cyclooxygenases-1 and -2 (COX-1 and COX-2) (Kim et al., 1998). In general, COX-1 is constitutively expressed in a wide range of tissues including the gastro-intestinal tract and plays a role in the tissue

homeostasis, e.g. maintenance of gastro-intestinal integrity (Singer et al., 1998). The inducible form, COX-2, which regulates prostaglandin synthesis, is overexpressed in several epithelial cancers and at sites of inflammation (Parker et al., 1997; Kim et al., 1998; Singer et al., 1998; Poligone and Baldwin, 2001; Martel-Pelletier et al., 2003). The expression of this enzyme is induced by various stimuli, e.g. TNF-α, IL-1β and phorbol esters, in a variety of cell lines.

Natural compounds, such as components of green tea, influence inflammation and cancer (Chen et al., 2003). EGCG, the major catechin of green tea, has many biological functions, including anti-inflammatory and chemopreventive effects (Lin and Lin, 1997; Ahn et al., 2004; Park and Surh, 2004). In China, Korea and Japan, where a large proportion of the population consumes green tea daily, the incidence of colon cancer is relatively low compared with that found in Western societies. EGCG interferes in several steps of inflammatory processes, e.g. in the synthesis of eicosanoids, and in the chemokine-mediated recruitment of PML to the injured site (Chen et al., 2002). Furthermore, EGCG was shown to suppress the maturation of murine DC through the inhibition of ERK, p38 kinase, JNK and the NF-κB signaling pathway (Ahn et al., 2004). *In vivo* studies show that green tea polyphenols decrease inflammation in animal models (Varilek et al., 2001).

Several reports have shown the benefits of EGCG in a variety of inflammatory conditions. In the present study, we evaluate the anti-inflammatory effects of EGCG *in vitro*. Human colon adenocarcinoma cell lines HT29 and T84 were stimulated with TNF-α and the potential of EGCG to improve gastro-intestinal inflammation, by modulating expression of cytokines and chemokines, was studied. Here we show that EGCG effectively modulates a number of mediators involved in different inflammatory diseases on protein and gene expression level. Our results suggest that EGCG may be of preventive and, furthermore, therapeutic value to treat intestinal inflammation.

RESULTS

Impact of EGCG on the viability of intestinal epithelial cells

The cytotoxicity of cell treatments and EGCG was evaluated by measuring the LDH activity in cell culture supernatants. Table 3 shows percentages of LDH released in HT29 and T84 cells. For HT29, the amount of released LDH was similar in the different treatments, indicating that TNF-α and EGCG did not affect the cell viability to a higher extent than TNF-α. This, however, might induce apoptosis. T84 cells treated with 25 μM of EGCG displayed higher LDH release than untreated cells, suggesting that EGCG might induce apoptosis.

Table 3. Determination of cell viability in HT29 and T84 cells treated with EGCG.

Cell viability	HT29 (% of LDH contents)	T84 (% of LDH contents)
Unstimulated cells	2.5 ± 0.0	1.3 ± 0.1
TNF-α (100 ng/ml)	5.8 ± 1.7	2.4 ± 0.4
EGCG (50 μM)	5.1 ± 1.3	0.6 ± 0.2 *
EGCG (25 μM)	2.3 ± 1.0	2.5 ± 0.7
TNF-α + EGCG (50 μM)	4.6 ± 0.4	1.9 ± 0.0
TNF-α + EGCG (25 μM)	6.3 ± 0.4	6.2 ± 0.7 *

Cells were stimulated with 100 ng/ml of TNF-α and incubated in presence or absence of varying concentrations of EGCG. After 24 hours, LDH was determined in culture supernatants immediately after harvest. Results are given as percentage of total LDH contents ± SD. Similar data have been obtained in at least two independent sets of experiments.

TNF-α induces the secretion of chemokines

TNF-α is an important pro-inflammatory mediator involved in gastro-intestinal inflammation and able to activate the synthesis of chemokines in epithelial cells (MacDermott, 1999). Therefore, we verified its effects on the production of IL-8 and MIP-3α in HT29 and T84 cell lines. Cells were cultured in the presence or absence of TNF-α for 24 hours and the secretion of these chemokines was examined. Unstimulated cells did not produce significant levels of IL-8 and MIP-3α. In contrast, TNF-α-activated cells demonstrated time- and dose-dependent synthesis of IL-8 and MIP-3α in HT29 (Fig. 4A and C). Similar data for the dose-dependent secretion of both chemokines were also obtained in T84 cells (data not shown). The time-dependent production of IL-8 and MIP-3α in TNF-α-stimulated T84 cells is

shown in Figure 4B and D. Compared to T84 cells, the production of IL-8 in activated HT29 cells was 1.5 fold increased after 24 hours. However, no significant differences were observed on the MIP-3α production between both cell lines (Fig. 4).

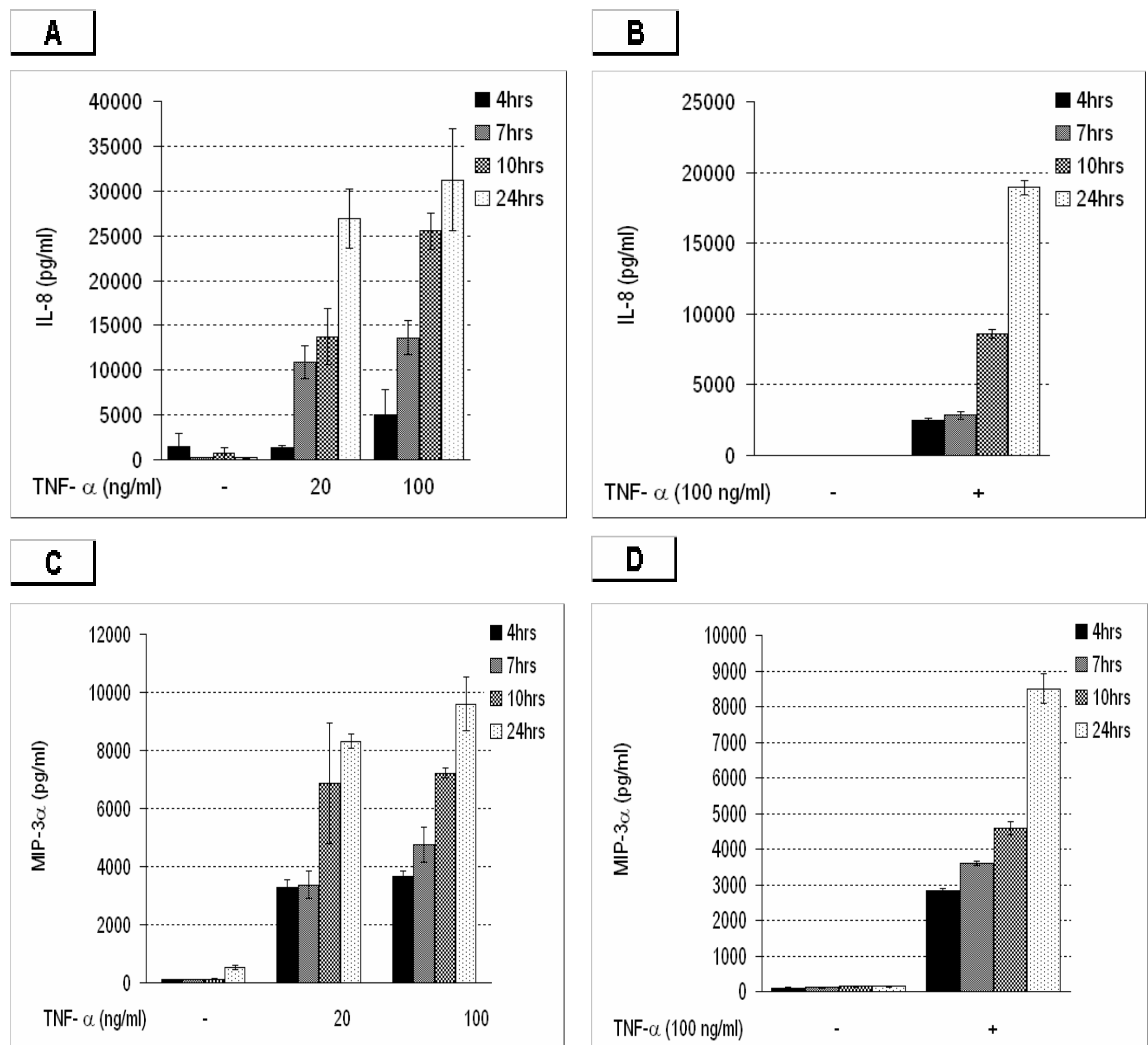


Figure 4: Induction of IL-8 and MIP-3α production by TNF-α-stimulated HT29 and T84 cells

Unstimulated and TNF-α-stimulated HT29 and T84 cells were cultured for 4 to 24 hours. IL-8 and MIP-3α secretion were measured in culture supernatants by ELISA. Representative data of one of three similar experiments are given. A and B, dose- and time-dependent increase of IL-8 production in HT29 and T84 cells, respectively. C and D, dose- and time-dependent increase of MIP-3α secretion in HT29 and T84 cells, respectively.

EGCG inhibits the production of IL-8 and MIP-3α in intestinal epithelial cells

The effects of EGCG on the IL-8 and MIP-3α secretion were investigated after 24 and 72 hours in TNF-α-stimulated HT29 and T84 cells. EGCG significantly reduced the production of IL-8 at 24 hours (Fig. 5A) and virtually abolished it at 72 hours (Fig. 5B). Increasing concentrations of EGCG gradually reduced IL-8 secretion in activated cells, indicating that the secretion of IL-8 from the cell to the culture medium was more affected in the presence of EGCG in HT29 than in T84 cells (Fig. 5C). This indicates a difference in the sensitivity of these cells to EGCG.

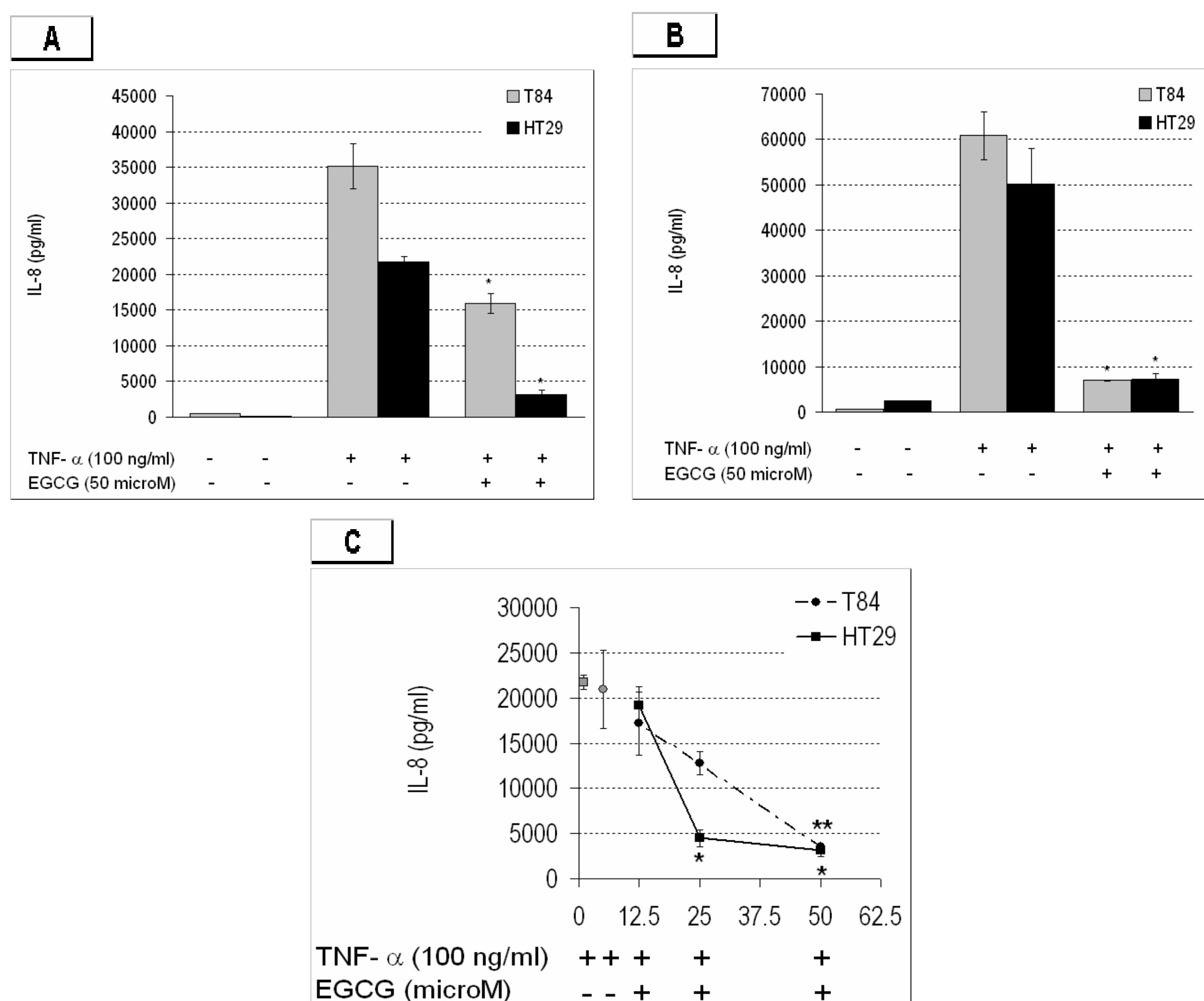


Figure 5: EGCG inhibited the production of IL-8 in epithelial cells

Levels of IL-8 in supernatants of unstimulated and TNF-α-stimulated HT29 and T84 cells cultured for 24 (A) and 72 (B) hours in the presence of 50 μM of EGCG. EGCG dose-dependently inhibited the synthesis of IL-8 in these epithelial cells (C). TNF-α-activated HT29 and T84 cells produced 21704 pg/ml and 20962 pg/ml IL-8, respectively. The secretion of IL-8 was determined by ELISA. * $p < 0.05$, ** $p < 0.01$. Representative data of one of three similar experiments are shown.

To investigate the impact of EGCG on MIP-3α secretion, TNF-α-activated cells were incubated with varying concentrations of EGCG for 24 hours. EGCG dose-dependently decreased the synthesis of MIP-3α in both cell lines (Fig. 6A). The production of MIP-3α was also investigated after 72 hours of culture (Fig. 6B). As expected, TNF-α strongly increased the synthesis of MIP-3α in both HT29 and T84 cells. This increase was significantly reduced in the presence of EGCG at a concentration of 50 μM.

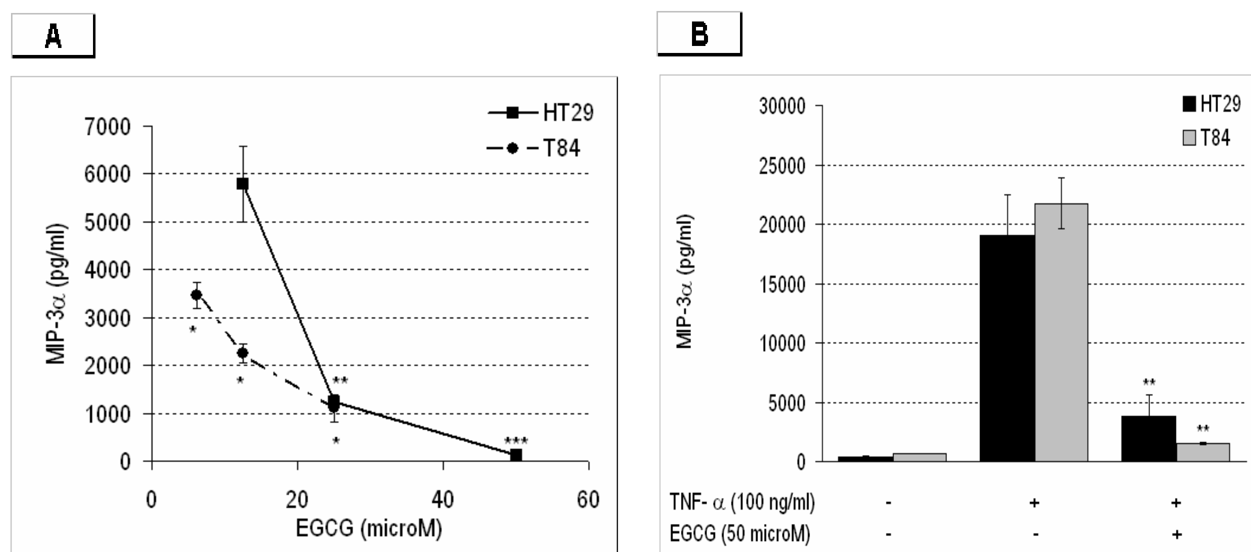


Figure 6: Effect of EGCG on TNF-α-induced MIP-3α production in HT29 and T84 cells

Cells were stimulated with 100 ng/ml of TNF-α in the presence of varying concentrations of EGCG for 24 hours (A). Unstimulated and stimulated cells were cultured for 72 hours in the presence of 50 μM of EGCG (B). The synthesis of MIP-3α was determined by ELISA. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Representative data of one of three similar experiments are shown.

EGCG inhibits the production of PGE_2 in epithelial cells

Since it is known that intestinal epithelial cells produce PGE_2 (Eckmann et al., 1997), we decided to investigated the influence of EGCG on its synthesis in activated HT29 cells. Unstimulated cells produced low amounts of PGE_2. Upon TNF-α stimulation a significant increase on the PGE_2 secretion was observed. Figure 7A shows that TNF-α dose-dependently augmented the secretion of PGE_2 after 24 hours. The highest amount of PGE_2 was obtained in cells stimulated in the presence of 100 ng/ml of TNF-α. In cells treated with 50 μM EGCG, PGE_2 synthesis was diminished by 80 ± 6% (data not shown). EGCG dose-dependently reduced the production of PGE_2 (Fig. 7B).

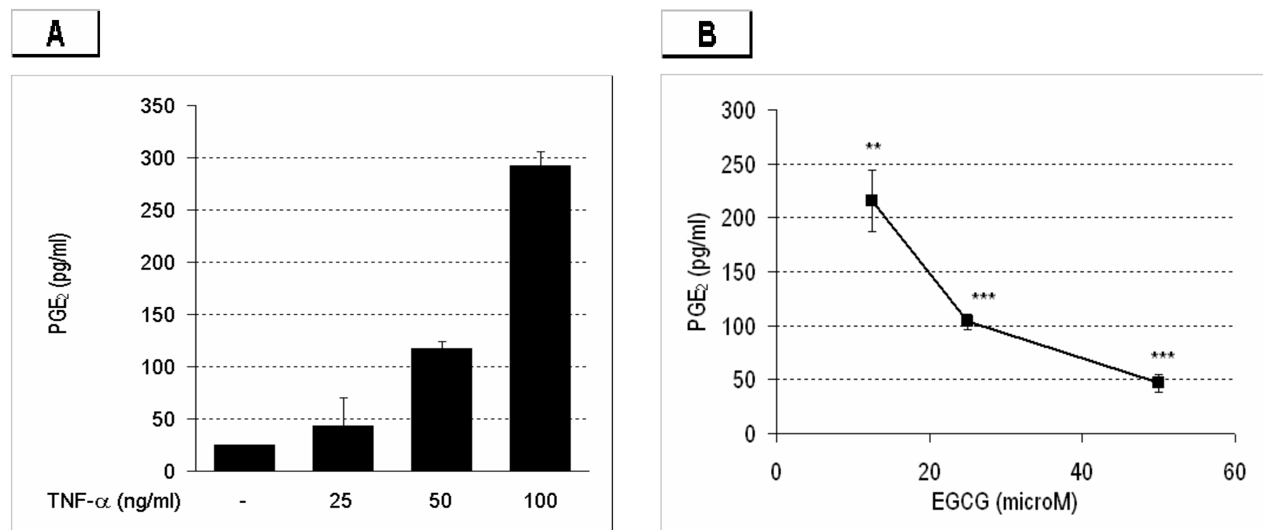


Figure 7: EGCG inhibited the secretion of PGE_2 in TNF-α-activated HT29 cells

HT29 cells were cultured with different concentrations of TNF-α for 24 hours (A). Stimulated cells were incubated with varying concentrations of EGCG for 24 hours (B). Activated cells produced 420 pg/ml of PGE_2. The production of PGE_2 was determined by ELISA. ** $p < 0.01$ and *** $p < 0.001$. Similar results were obtained in two other series of experiments.

EGCG modulates genes involved in inflammatory responses

In order to clarify whether EGCG modulates the expression of TNF-α-induced genes, we used quantitative RT-PCR. Since activated HT29 and T84 cells up-regulated inflammatory genes within 2-24 hours (Fig. 8), the influence of EGCG on these genes was analyzed after 6 hours of culture. Basal gene expression levels in unstimulated HT29 cells were different, with weakly (e.g. TNF-α, MIP-2, GRO-α, IL-1α, IL-1β, IP-10), moderately (IL-8, MIP-3α, GRO-γ, PGES, MMP-14, RANK, IEX-1L, CFb) and abundantly (e.g. COX-2, 5-LOX, β 5 integrin, RANTES, FRA-1) expressed genes (Fig. 9). In T84 cells, however, basal gene expression levels differed from those of HT29 cells with respect to moderately (e.g. IL-8, MIP-3α, GRO-γ, PGES, MMP-14, RANK, IEX-1L, RANTES, CFb) and abundantly (e.g. COX-2, 5-LOX, β 5 integrin, FRA-1) expressed genes (data not shown).

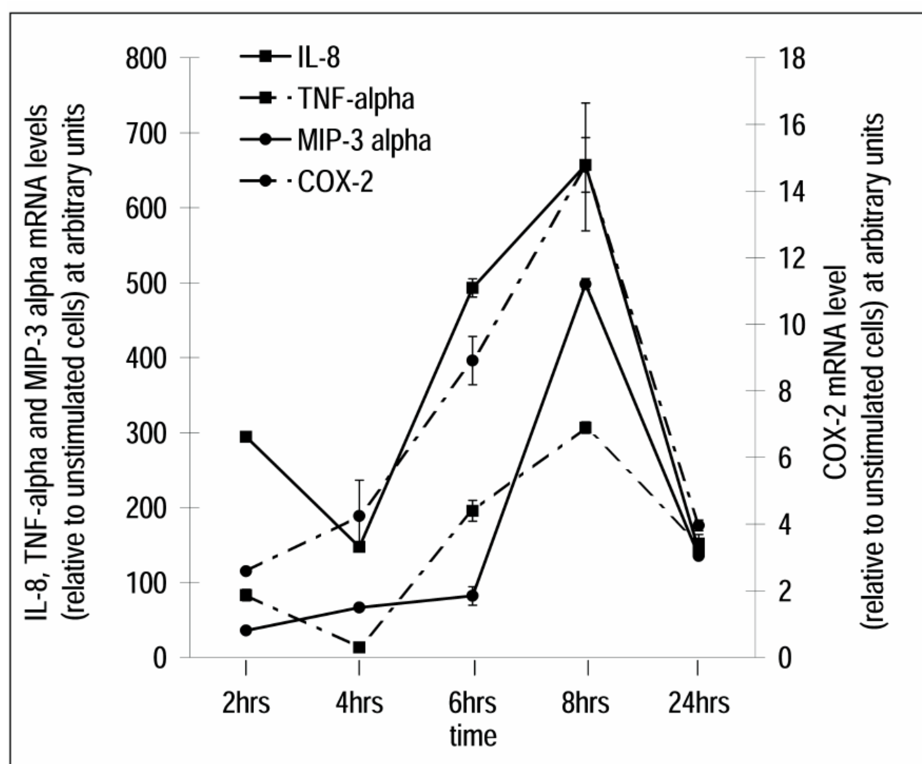


Figure 8: Time-dependent up-regulation of specific genes in HT29 cells

Cells were cultured without or with 100 ng/ml of TNF-α for 6 hours. RNA was isolated and gene expression levels of IL-8, TNF-α, MIP-3α and COX-2 were measured using the quantitative RT-PCR. Values are expressed relative to unstimulated cells at arbitrary units ± SD. Similar results were obtained in two other series of experiments.

TNF-α induced a substantial increase in mRNA levels of cytokines and chemokines (TNF-α, IL-8, MIP-2, MIP-3α, GRO-α, GRO-γ, IP-10), in addition to COX-2. However, mRNA levels of other genes, including 5-LOX, RANK, and β 5 integrin, were unaltered by TNF-α stimulation. EGCG (50 μM) reduced the mRNA levels of IL-8, TNF-α, MIP-3α, MIP-2, GRO-α, GRO-γ and COX-2 by 40 to 85% in both cell lines (Fig. 10). This effect was specific, but not restricted, to these genes. Conversely, EGCG did not augment the expression of TNF-α-induced genes. TNF-α strongly augmented the expression of IL-8 mRNA levels in HT29 and T84 cells, while it moderately and weakly increased the expression of TNF-α, MIP-3α, and GRO-γ mRNA (Fig. 10A, B and E), COX-2 and GRO-α mRNA (Fig. 10C and D), respectively. Notably, these genes were significantly down-regulated by EGCG, an observation which is consistent with our results of produced chemokines (IL-8 and MIP-3α) (Fig. 5 and 6).

Finally, the dose-dependent impact of EGCG on gene expression was evaluated. In HT29 and T84 cells, EGCG down-regulated IL-8, TNF-α, MIP-3α and COX-2 mRNA levels in the concentration range of 6.25 to 50 μM (Fig. 11). In T84 cells, EGCG at 12.5 and 25 μM did

not have any significant effect on IL-8 mRNA levels, although a trend for down-regulation was observed (Fig. 11B). In summary, expression levels of tested chemokines were significantly diminished following EGCG treatment in these cell lines.

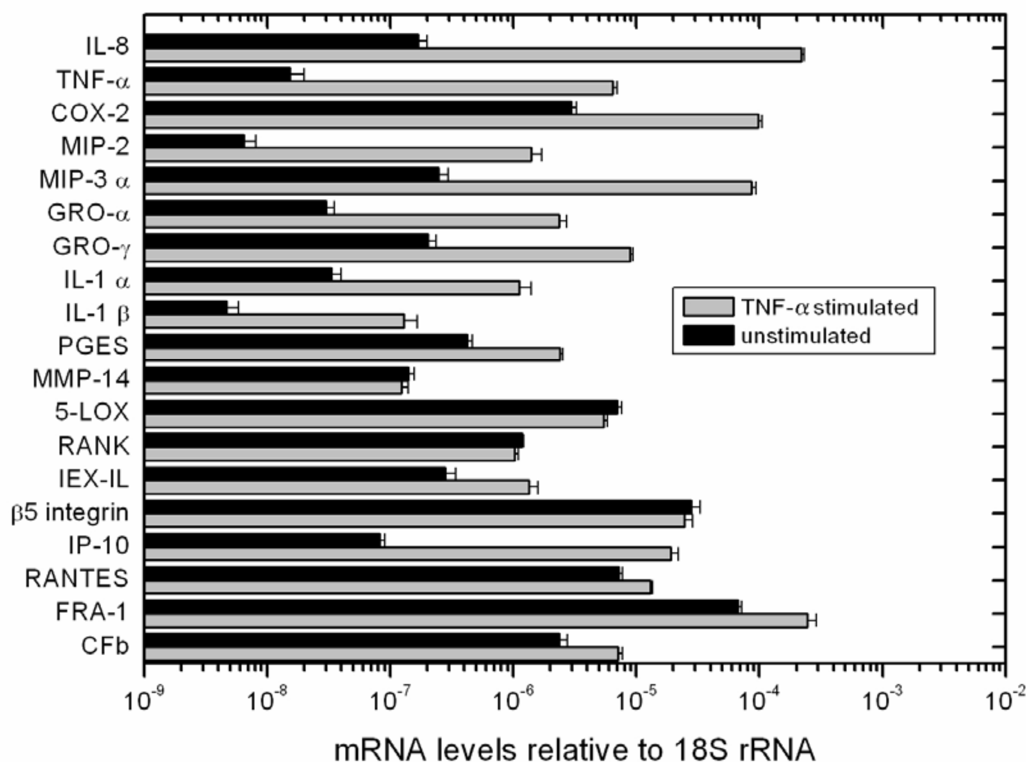


Figure 9: Expression levels of selected genes in HT29 cells

RNA of unstimulated and TNF-α-activated HT29 cells which were cultured for 6 hours were isolated and the levels of mRNA of the indicated genes determined by quantitative RT-PCR. Values are expressed relative to 18S rRNA and are indicated as means ± SD of 2 to 4 experiments.

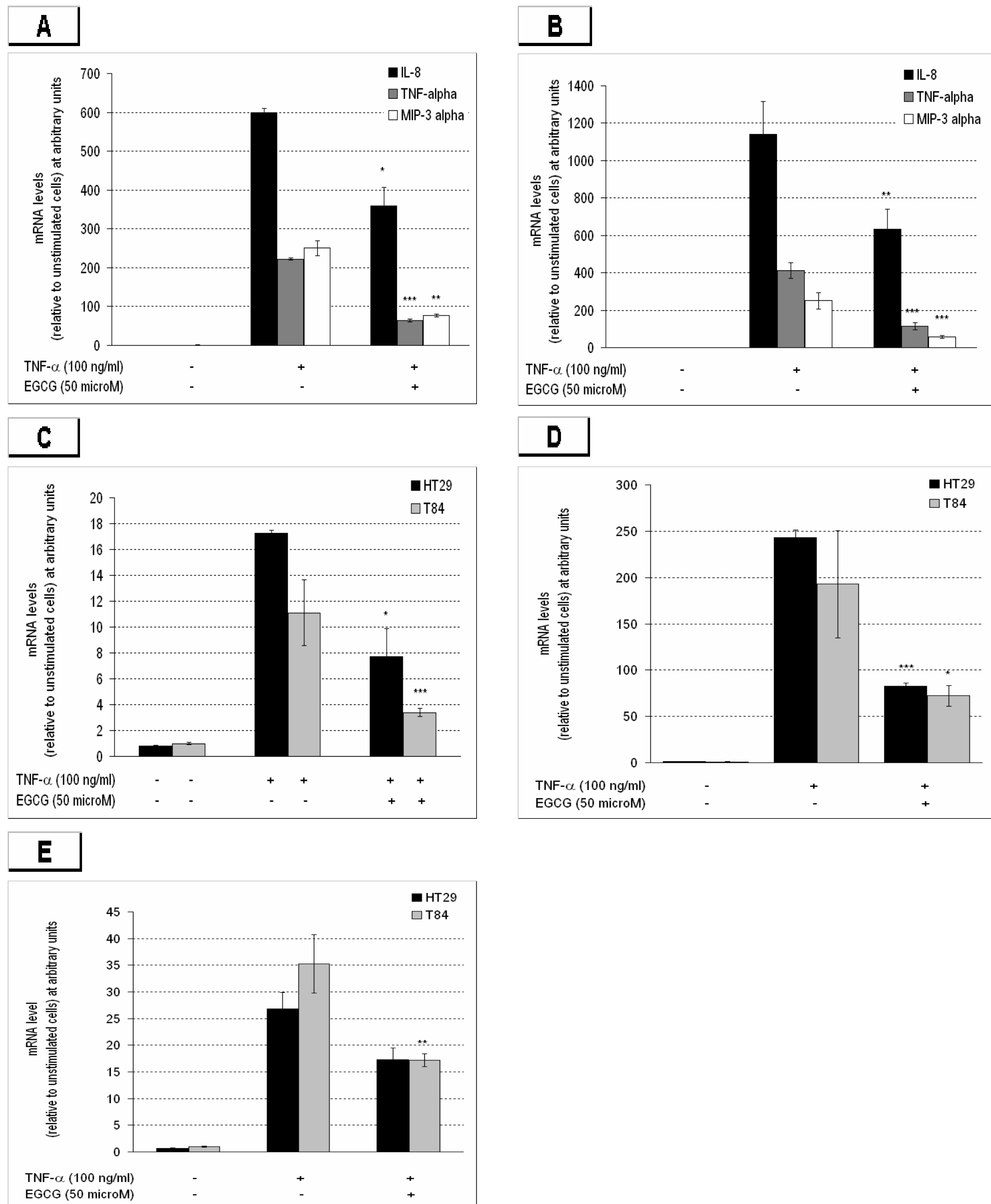


Figure 10: EGCG modulated the expression level of inflammatory genes

Activated HT29 and T84 cells were cultured in the presence of 50 μM of EGCG for 6 hours. The effects of EGCG on mRNA expression levels of IL-8, TNF-α and MIP-3α in HT29 and T84 cells, respectively, were analyzed (A and B). The influence of EGCG on COX-2, GRO-α and GRO-γ mRNA levels in HT29 and T84 cells, respectively, are given (C, D and E). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Representative data of one of four similar experiments are shown.

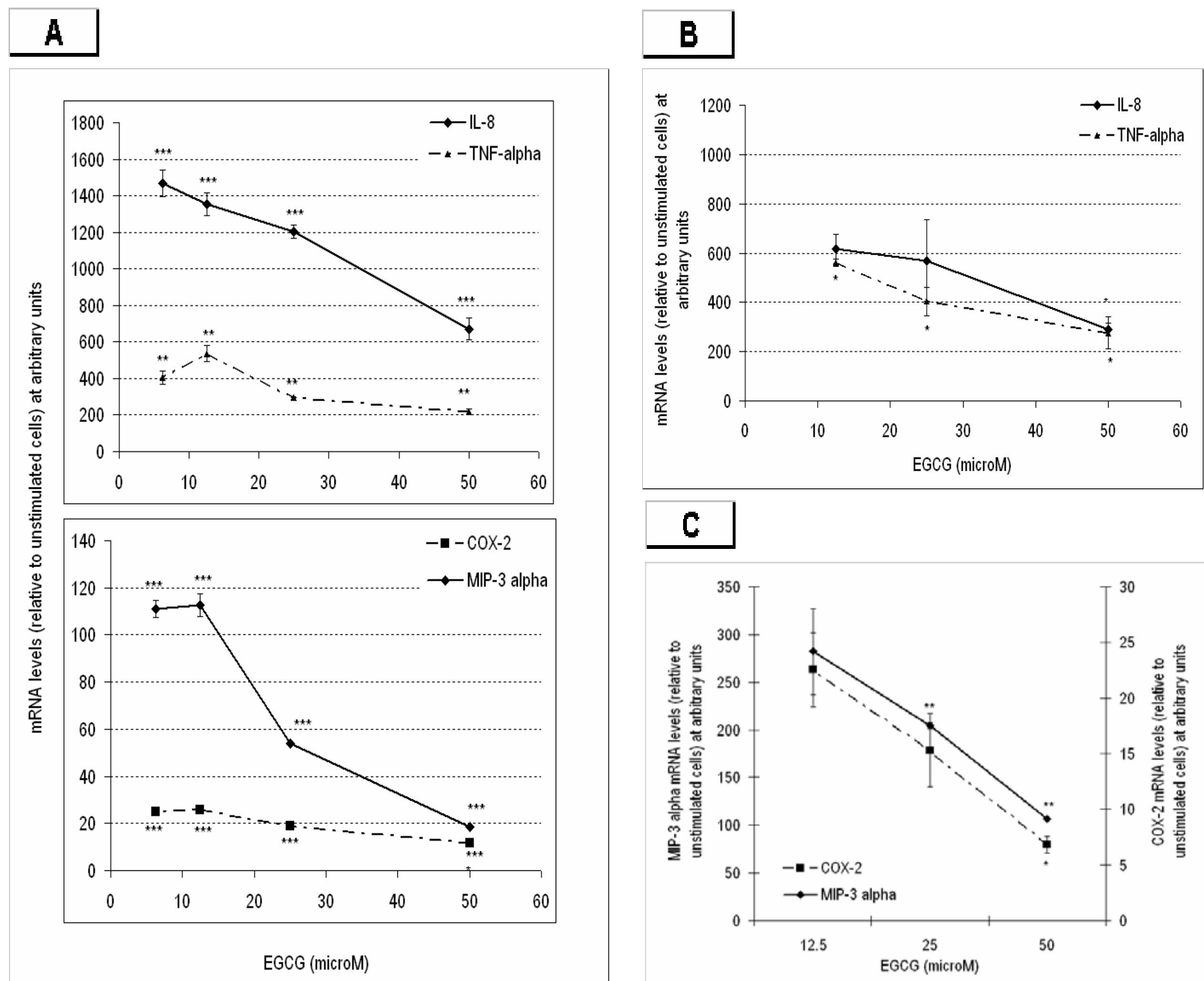


Figure 11: EGCG dose-dependently down-regulated some inflammatory genes

TNF-α-stimulated HT29 (A) and T84 cells (B and C) were cultured in the presence of varying concentrations of EGCG for 6 hours. The effect of EGCG on the mRNA level of IL-8, TNF-α, MIP-3α and COX-2 is given. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Representative data of one of three similar experiments ± SD are shown.

DISCUSSION

In this report, we present evidence that EGCG beneficially affects the inflammatory response in the colon adenocarcinoma cell lines HT29 and T84. These cell lines were used as models for intestinal mucosal diseases and have different origins and phenotypes. *In vitro*, differentiated HT29 cells express characteristic features of mature intestinal cells, such as microvilli, small intestinal digestive enzymes, lipid droplets, primary and secondary lysosomes (Velcich et al., 1995). T84 is a transplantable human carcinoma cell line derived from a lung metastasis of colon carcinoma (Murakami and Masui, 1980) which possess secretory capacity such as small intestinal crypt cells.

Our experimental approach dealt with the identification of the anti-inflammatory function of EGCG in TNF-α-activated colon adenocarcinoma cell lines. The effects of EGCG were determined at two levels: the expression of genes involved in inflammation and the production of chemokines (IL-8 and MIP-3α) and PGE_2. EGCG showed potent effects on genes of the inflammatory pathway, including those of the cytokine/chemokine network (e.g. IL-8, TNF-α, MIP-3α, MIP-2, GRO-α and GRO-γ). CXC chemokines, such as IL-8, MIP-2, GRO-α and GRO-γ, are responsible for chemoattraction of neutrophils, while CC chemokines (e.g. MIP-3α) play a role in the recruitment of lymphocytes and DC to the inflamed tissue. Cytokines and chemokines are induced by TNF-α stimulation and are abundantly expressed and secreted in IBD (Fiocchi, 1997b; Fiocchi, 1997a; Ajuebor and Swain, 2002; Banks et al., 2003; Kaser et al., 2004; Papadakis, 2004). We demonstrated that EGCG markedly diminished the expression and secretion of IL-8 and MIP-3α in colon epithelial cells.

IBD and colon cancer are associated with an increased activity of intestinal immune cells, which augments the production of pro-inflammatory cytokines, including TNF-α. Therefore, TNF-α was used to induce an inflammatory state *in vitro*. TNF-α is involved in the mediation of the sustained inflammatory response and high amounts of this cytokine can be found in IBD patients (Dionne et al., 1998; D'Haens, 2003). The expression and production of IL-8 were studied in macrophages isolated from normal and inflamed colonic tissue resected for IBD (Grimm et al., 1996). Macrophages from IBD expressed more IL-8 mRNA than those from the normal mucosa, and lipopolysaccharide treatment further increased it. In addition, the recruitment of macrophages may be responsible for the IL-8 secretion which leads to neutrophil attraction in IBD (Grimm et al., 1996). This suggests that in the intestine activated macrophages migrate into the epithelium and secrete IL-8 which recruits PML to the injured site. This is supported by our macrophage *in vitro* model (data not shown) where RAW 264.7 cells were stimulated with LPS in the presence or absence of EGCG. In this study, we have identified EGCG as an inhibitor of IL-8. Moreover, after TNF-α stimulation, epithelial

cells also produce IL-8, and consequently, recruit PML. The inhibition of the expression and production of IL-8, either by EGCG or other natural compounds (i.e. phenolic compounds), interferes with the recruitment of PML and, thus, impedes progression and aggravation of inflammation (Sugimoto et al., 2002; Park and Surh, 2004). In conclusion, EGCG has the potential to reduce the IL-8 production in both epithelial cells and macrophages (Fig. 12).

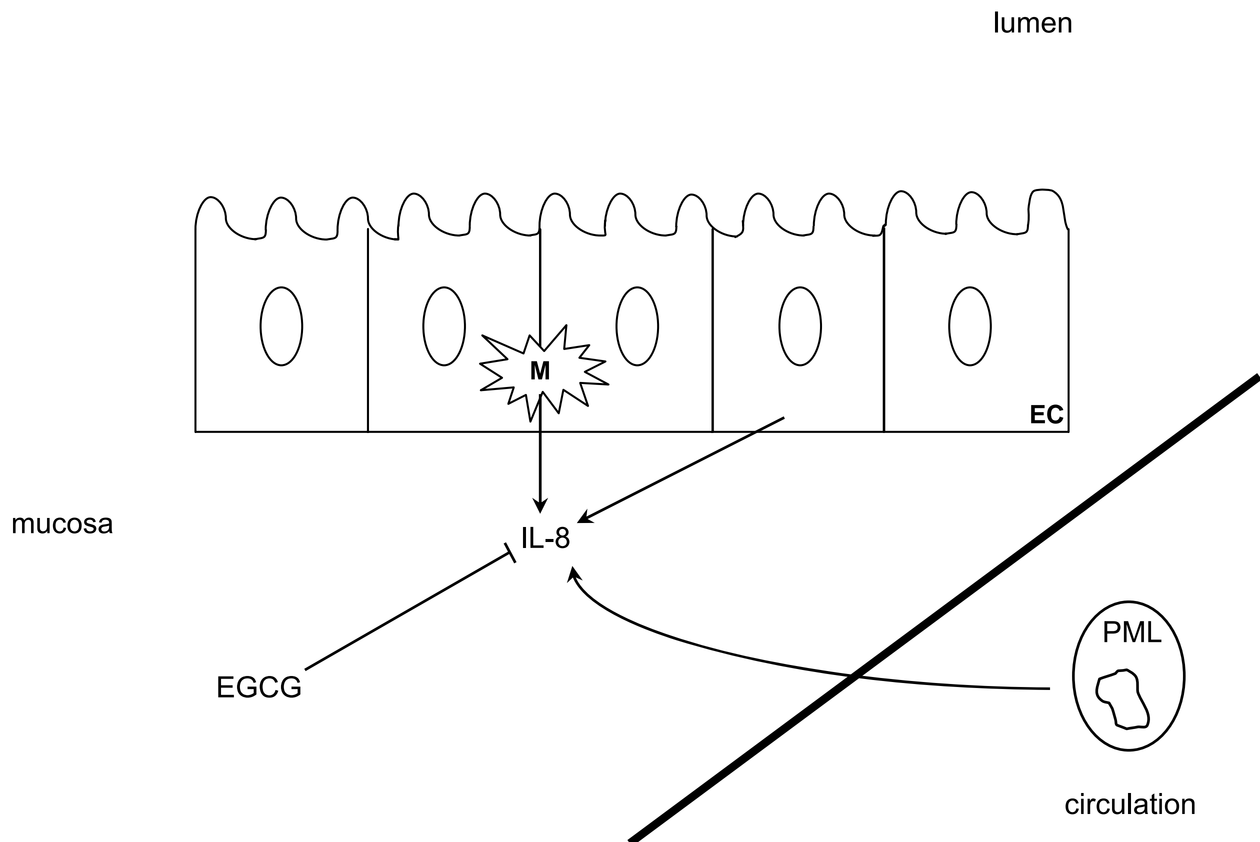


Figure 12: Crosstalk between epithelial cells (EC) and macrophages (M) in intestinal inflammation
Macrophages migrate into the epithelium and secrete IL-8. Activated epithelial cells also produce IL-8. These enhance on IL-8 is responsible to chemoattract PML from the circulation, increasing the inflammatory response. EGCG inhibit the synthesis of IL-8, and therefore, the recruitment of PML, reducing inflammation.

With regard to MIP-3α, it is synthesized by the human colon epithelial cells and therefore by HT29 cells which also express CCR6, the cognate receptor for MIP-3α (Izadpanah et al., 2001). MIP-3α mRNA and protein levels were found to be increased in colonic tissues from patients with IBD (Kwon et al., 2002). In the present study, EGCG inhibited the synthesis of MIP-3α in colon cancer cell lines and also down-regulated gene expression. Via MIP-3α production, intestinal epithelial cells recruit DC and memory T cells to the site of inflammation. Whereas the normal intestinal mucosa is home of dendritic and T cells, a specific T cell population immigrates during IBD and contributes to the generation of inflammation (Fiocchi, 1998). Therefore, an excess of these cells in the inflamed intestine

should be avoided. By reducing MIP-3α production, EGCG could prevent homing of these cells and consequently IBD symptoms.

Furthermore, we observed that EGCG inhibits PGE_2 production. In the gastro-intestinal environment, prostaglandins are important regulators of gastro-intestinal fluid secretion. In food allergies or after invasion of microorganisms, water secretion and TNF-α-induced electrolyte production lead to an increase of Ca^{2+}-dependent PGE_2 in the intestinal epithelium. Exogenous PGE_2 up-regulated IL-8 gene expression and protein production in human colonic epithelial cells (Yu and Chadee, 1998). PGE_2 production was increased in colonic fibroblasts and in IBD patients. Intestinal fibroblasts, beside epithelial cells, could be targets for PGE_2 and sites of colonic prostanoid biosynthesis *in vivo* (Kim et al., 1998). The expression of COX-2 has also been observed after Salmonella infection in intestinal epithelial cells (Singer et al., 1998), and COX-2 protein was reported to be expressed in three of eight colon cancer cell lines, including HT29 (Parker et al., 1997). Our results demonstrated that EGCG diminished pro-inflammatory PGE_2 production and expression in HT29 cells. Studies have shown that EGCG targets COX-2 by inhibiting the PGE_2 production and possibly reduces the risk of colon cancer and inflammation in humans (August et al., 1999; Hong et al., 2001; Park and Surh, 2004). Due to differences between HT29 and T84 cells, TNF-α-stimulated T84 cells did not produce detectable amounts of PGE_2 and COX-2 protein (data not shown). With regard to this observation, in HT29 cells, EGCG had shown an influence on the three parameters tested: COX-2 gene, COX-2 protein expression (data not shown) and PGE_2 generation, whereas in T84 cells, only the COX-2 mRNA levels were affected by EGCG.

Quantitative RT-PCR analysis identified several genes that were induced in TNF-α-stimulated HT29 and T84 cells, including IL-8, TNF-α, MIP-3α, MIP-2, GRO-α and GRO-γ. These genes were down-regulated by EGCG and have NF-κB regulatory binding sites. Consequently, they are regulated by the transcription factor NF-κB. Similar to the effects observed for IL-8, the expression levels of MIP-2, GRO-α and GRO-γ were also diminished by EGCG in both cell lines. These chemokines, like IL-8, act through the chemokine receptor CXCR2 (Murphy et al., 2000) and are also elevated after TNF-α stimulation (Fig. 10D and E). The concerted effect of EGCG on four chemokines binding to a common receptor indicates that this flavonoid impedes most or all biological activities that are mediated by CXCR2. Furthermore, 5-LOX, an enzyme expressed in differentiated HT29 cells (Cortese et al., 1995) and responsible for the synthesis of leukotrienes, was also expressed in HT29 and T84 cell lines, but no differences was observed between unstimulated and TNF-α-activated cells (Fig. 9). In our experiments, the expression of 5-LOX was not influenced by EGCG (data not shown). A possible explanation for this observation is that TNF-α is not able to induce 5-LOX.

Collectively, we provide evidence that EGCG attenuates the inflammatory response in the colon adenocarcinoma cell lines HT29 and T84 by inhibiting the production of chemokines and PGE_2. However, this study only refers to the effect of EGCG in the *in vitro* gastro-intestinal inflammation and, thus, it should be followed by *in vivo* experiments to consolidate this statement. In addition, *in vivo* studies will also corroborate the use of EGCG in preventing and attenuating gastro-intestinal disorders.

Chapter 2

Effects of EGCG on the gene expression profile of activated human colon cancer cells HT29

ABSTRACT

As shown in the preceding chapter, EGCG exibits anti-inflammatory activities in human colon cancer cells. The aim of this study was to monitor the effects of EGCG on global gene expression in activated HT29 cells and to infer on its presumably wide effect on cellular physiology. Cells were stimulated with or without TNF-α (100 ng/mL) in the presence or absence of various concentrations of EGCG, and gene expression changes were determined after 6 hours of culture. TNF-α significantly influenced 700 of 14500 genes tested. These were sorted by their change factors and only those > 0.5 (up-regulated) and < -0.5 (down-regulated) were analysed (*P* value < 0.01). The gene chip analysis began with 179 genes where 151 were up-regulated and 28 down-regulated by TNF-α; however, only 78 were described in this paper. Analyses revealed that EGCG alone mildly but significantly modulated the expression of genes involved in immune responses and inflammation, confirming previous results. In addition, genes involved in apoptosis, in growth factor signalling, and in transcription were also regulated by EGCG. TNF-α-stimulated cells markedly up-regulated chemokines, apoptotic signals, and cell adhesion molecules including ICAM-1. "Inflamed" cells exposed to EGCG reversed inflammatory responses induced by TNF-α, increasing survival signals and decreasing the production of chemokines and apoptotic signals. Interestingly, not all chemokines were altered by EGCG (e.g. granulocyte chemotactic protein - GCP-2). Furthermore, components of the extracellular matrix (ECM) including matrix metalloproteinase (MMP)-7, collagen and laminin 5 were up-regulated by EGCG, suggesting that EGCG might induce tissue remodelling and assist in the regeneration of the epithelium. Moreover, several components of specific signalling pathways such as NF-κB, AP1, MAPK and PI3K were affected by EGCG. This study has confirmed previous observations concerning the effects of EGCG on chemokines and extended knowledge on pathways or other processes affected by EGCG, including TNF/TNFR1 and growth factor signalling in addition to cell adhesion and cell death (caspase cascade). Furthermore, potential new leads to genes affected by EGCG that could play a role in the prevention of intestinal inflammation were identified such as those involved in barrier integrity.

INTRODUCTION

As shown in the Chapter 1, EGCG acts as an anti-inflammatory agent in intestinal inflammation *in vitro*; however, the mode by which EGCG exerts its action is not completely elucidated. New techniques for the large-scale analysis of gene expression in host cells have opened an array of opportunities for studying cellular responses. In particular, global gene expression profiling using microarrays offers new opportunities to study and identify the effects of food components on the transcriptional level. By using cDNA microarrays the expression of thousand of genes can be studied in one experiment, in addition, leads to pathwas can be identified.

The aim of this study was to use genome-wide information on gene expression profile induced by TNF-α in colon cancer cells and to elucidate additional features of EGCG beside its anti-inflammatory effect.

RESULTS AND DISCUSSION

We have analysed the transcriptional response of a human colon adenocarcinoma cell line, HT29, which can be induced to express inflammatory features *in vitro*. EGCG, a catechin found in abundance in green tea, was used to reverse TNF-α-induced inflammation. Two thousand one hundred and fifty one genes of 14500 present in the chip were significantly regulated by at least one of the treatments. TNF-α significantly influenced 700 genes. Accordingly to its impact, genes were sorted by their change factors and only those > 0.5 (up-regulated) and < -0.5 (down-regulated) were analysed (P value < 0.01). The analysis began with 179 genes where 151 were up-regulated and 28 down-regulated by TNF-α (Table 4). Among them, EGCG alone up-regulated 65 genes, down-regulated 45 genes, and did not affect 69. In the TNF-α/EGCG treated group, the number of genes up- or down-regulated by EGCG can also be visualized in Table 4 where red and green numbers represent up- and down-regulated genes, respectively.

Table 4. List of TNF-α-induced genes that were sensitive to EGCG

AFFY_ID	DESCR	Gene Symbol	TNF-α[a]	TNF-α + EGCG (μM)[a]				EGCG (μM)[a]				Biological Process
				6.25	12.5	25	50	6.25	12.5	25	50	
201631_s_at	immediate early response 3	IER3 or DIF2	2.75	2.49	2.03	1.80	1.50	-0.29	-0.31	-0.20	0.04	immediate early
201739_at	serum/glucocorticoid regulated kinase	SGK1	2.07	2.33	2.38	3.98	4.41	0.32	0.38	1.15	2.29	immediate early; signalling:kinase
209716_at	colony stimulating factor 1 (macrophage)	CSF1 or MCSF	7.65	6.37	6.61	5.04	4.88	0.18	-0.07	0.41	0.12	immune reponse
201925_s_at	decay accelerating factor for complement (cd55)	DAF or CD55	2.12	2.53	3.14	3.48	3.28	0.38	0.41	1.53	1.36	immune reponse
207375_s_at	interleukin 15 receptor, alpha	IL15RA	1.39	1.02	1.50	1.01	1.34	0.06	0.03	0.11	0.45	immune reponse
204863_s_at	interleukin 6 signal transducer	IL6ST or gp130	1.51	1.98	2.03	1.58	1.20	0.13	-0.05	0.12	-0.15	immune reponse
203320_at	lymphocyte adaptor protein	LNK	0.82	0.52	0.90	0.67	0.99	-0.11	0.03	-0.10	0.53	immune reponse
207339_s_at	lymphotoxin beta (tnf superfamily, member 3)	LTB or TNFC	10.05	8.03	6.95	5.69	3.59	-1.28	-0.28	0.16	-0.03	immune reponse
205476_at	chemokine (c-c motif) ligand 20	CCL20; MIP3a or LARC	429.01	405.97	331.36	218.70	111.60	-0.35	-0.35	-0.35	-0.35	immune reponse: chemokine
823_at	chemokine (c-x3-c motif) ligand 1	CX3CL1 or fractalkine	3.45	2.65	3.01	2.18	1.55	-0.24	-0.42	-0.27	-0.08	immune reponse: chemokine
204470_at	chemokine (c-x-c motif) ligand 1	GROa; MGSAa or CXCL1	34.56	23.17	22.04	19.93	9.37	-0.69	-0.72	-0.37	0.14	immune reponse: chemokine
204533_at	chemokine (c-x-c motif) ligand 10	CXCL10 or IP10	44.60	25.82	26.46	18.10	8.07	-0.07	-0.12	-0.26	-0.21	immune reponse: chemokine
211122_s_at	chemokine (c-x-c motif) ligand 11	CXCL11 or IP9	7.23	4.71	5.96	6.37	4.04	-0.17	-0.02	-0.02	0.10	immune reponse: chemokine
209774_x_at	chemokine (c-x-c motif) ligand 2	CXCL2; GROb or MIP2a	22.18	15.17	13.18	8.84	5.47	-0.06	-0.06	-0.06	0.45	immune reponse: chemokine
207850_at	chemokine (c-x-c motif) ligand 3	GROg; MIP2b or CXCL3	17.16	12.88	10.27	9.84	4.53	-0.51	-0.05	-0.13	-0.40	immune reponse: chemokine
206336_at	chemokine (c-x-c motif) ligand 6	GCP2 or CXCL6	4.89	5.78	4.49	4.85	4.56	1.50	0.87	1.29	0.46	immune reponse: chemokine
211506_s_at	interleukin 8	IL-8; GCP1 or CXCL8	429.06	418.96	440.70	409.60	318.95	0.50	0.00	0.31	2.82	immune reponse: chemokine
211676_s_at	interferon gamma receptor 1	IFNGR1 or CD119	2.75	3.50	4.08	2.87	2.87	0.26	0.15	0.37	0.75	immune reponse: IFN
201642_at	interferon gamma receptor 2	IFNGR2	1.03	0.98	1.16	1.07	0.93	0.09	0.00	-0.10	0.12	immune reponse: IFN
219209_at	interferon induced with helicase c domain 1	IFIH1	1.99	1.51	1.71	0.66	-0.06	-0.07	-0.27	-0.87	-1.68	immune reponse: IFN
202531_at	interferon regulatory factor 1	IRF1	1.37	1.33	1.50	0.91	0.60	-0.12	-0.20	-0.34	-0.27	immune reponse: IFN
204698_at	interferon stimulated gene 20kda	ISG20	3.18	2.77	1.70	3.91	3.48	-0.34	-0.15	0.40	1.00	immune reponse: IFN
208965_s_at	interferon, gamma-inducible protein 16	IFI16	2.15	1.48	1.13	0.17	-0.46	-0.03	-0.16	-0.84	-1.33	immune reponse: IFN
209417_s_at	interferon-induced protein 35	IFP35 or IFI35	0.99	0.64	0.45	0.12	-0.19	-0.10	-0.32	-0.53	-0.56	immune reponse: IFN
204747_at	interferon-induced protein with tetratricopeptide repeats 3	IFIT3	1.71	0.83	0.91	-0.16	-0.92	-0.11	-0.40	-1.13	-1.70	immune reponse: IFN
203596_s_at	interferon-induced protein with tetratricopeptide repeats 5	IFIT5	2.04	1.22	0.78	0.07	-0.25	0.02	-0.06	-0.35	-0.42	immune reponse: IFN
202270_at	guanylate binding protein 1, interferon-inducible, 67kda	GBP1	7.00	4.62	4.21	2.62	2.79	-1.35	0.10	-0.26	-0.02	immune reponse: IFN
203153_at	interferon-induced protein with tetratricopeptide repeats 1	IFIT1 or IFI56	0.58	0.16	0.25	-1.07	-2.86	-0.12	-0.49	-1.73	-4.59	immune reponse: IFN
213191_at	tir domain containing adaptor inducing interferon-beta	TRIF or TICAM1	1.81	1.61	1.91	3.89	1.79	0.14	0.43	0.95	0.47	immune reponse: IFN
208729_x_at	major histocompatibility complex, class i, b	HLA-B	0.82	0.78	1.02	1.06	0.71	0.19	0.02	0.10	0.27	immune reponse: MHC
211799_x_at	major histocompatibility complex, class i, c	HLA-C	0.80	0.95	1.13	1.11	0.88	0.25	0.08	0.13	0.15	immune reponse: MHC
200905_x_at	major histocompatibility complex, class i, e	HLA-E or HLA-6.2	0.62	0.74	0.58	0.72	0.48	0.00	-0.18	-0.03	-0.08	immune reponse: MHC
221875_x_at	major histocompatibility complex, class i, f	HLA-F	0.60	0.60	0.48	0.51	0.43	0.18	0.09	-0.07	0.07	immune reponse: MHC
203828_s_at	natural killer cell transcript 4	NK4 or IL32	32.06	31.07	25.87	32.91	34.53	0.00	0.00	0.00	0.00	immune response
202086_at	myxovirus (influenza virus) resistance 1	Mx1 or IFI78	1.46	0.76	0.87	-0.09	-1.22	0.01	-0.37	-1.21	-2.70	immune response: IFN target
201648_at	janus kinase 1 (a protein tyrosine kinase)	JAK1	0.58	0.63	0.95	0.72	0.76	0.16	0.11	0.18	0.10	immune response: JAK/STAT
209969_s_at	signal transducer and activator of transcription 1, 91kda	STAT1	0.66	0.45	0.26	-0.41	-1.05	0.01	-0.44	-0.82	-1.27	immune response: JAK/STAT
204748_at	prostaglandin-endoperoxide synthase 2 (cyclooxygenase)	COX2 or PGHS2	21.05	20.36	24.41	16.40	11.68	-0.10	-0.19	0.30	0.34	immune response: PG
209792_s_at	kallikrein 10	KLK10	4.62	4.51	4.53	7.25	8.15	-0.28	-0.14	0.15	0.84	immune response: plasma protease
204549_at	inhibitor of kappa light polypeptide gene enhancer in b-cells, kinase epsilon	IKBKE or IKKe	0.80	0.86	1.00	0.67	0.40	0.16	0.11	0.06	0.14	immune response: TF NFKB
210458_s_at	traf family member-associated nfkb activator	TANK	0.63	1.09	1.16	0.84	0.49	-0.06	0.10	0.22	0.10	immune response: TF NFKB
206036_s_at	v-rel reticuloendotheliosis viral oncogene homolog	REL	0.62	0.59	1.16	0.70	0.34	0.10	0.03	0.16	0.16	immune response: TF NFKB
205205_at	v-rel reticuloendotheliosis viral oncogene homolog b	RELb	5.72	5.99	6.58	5.99	4.32	0.16	0.08	0.06	0.38	immune response: TF NFKB
209239_at	nuclear factor of kappa light polypeptide gene enhancer in b-cells 1 (p105)	NFkB1	1.40	1.27	1.61	1.31	1.16	0.08	0.00	0.05	0.05	immune response: TF NFKB
209636_at	nuclear factor of kappa light polypeptide gene enhancer in b-cells 2 (p49/p100)	NFkB2	8.88	13.83	13.28	11.22	8.29	0.00	0.00	0.00	0.10	immune response: TF NFKB
201502_s_at	nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, alpha	NFKBIA or IkBa	6.76	6.58	5.36	6.37	4.36	-0.31	-0.34	-0.26	-0.11	immune response: TF NFKB
214448_x_at	nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, beta	NFkBIb or IkBb	1.78	1.85	1.78	2.35	1.73	0.88	0.36	0.58	1.14	immune response: TF NFKB
203927_at	nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, epsilon	NFkBIe or IkBe	3.10	3.58	3.73	3.86	3.82	0.14	0.18	0.26	0.56	immune response: TF NFKB
204908_s_at	b-cell cll/lymphoma 3	BCL3	4.94	5.73	5.30	5.78	3.20	0.10	0.02	0.31	0.52	TF NFkB: survival

Table 4. List of TNF-α-induced genes that were sensitive to EGCG (CONTINUED)

212312_at	bcl2-like 1	BCL2L1	0.55	0.44	0.60	0.40	0.52	0.41	0.45	0.43	0.93	apoptosis: inhibitor
210538_s_at	baculoviral iap repeat-containing 3	BIRC3 or API2	24.30	24.03	30.34	21.45	20.60	-0.04	-0.08	-0.25	0.28	apoptosis: inhibitor
217996_at	pleckstrin homology-like domain, family a, member 1	PHLDA1 or TDAG51	1.75	1.76	2.11	2.08	2.72	0.01	0.12	0.52	1.35	apoptosis
209939_x_at	casp8 and fadd-like apoptosis regulator	CFLAR or casper	0.61	0.57	0.45	0.78	0.79	-0.07	-0.16	-0.03	0.06	apoptosis
209790_s_at	caspase 6, apoptosis-related cysteine protease	CASP6	-1.04	-0.97	-1.28	-1.11	-1.23	-0.27	-0.61	-0.40	-0.51	apoptosis
200798_x_at	myeloid cell leukemia sequence 1 (bcl2-related)	MCL1	1.23	1.32	1.67	1.46	1.41	0.28	0.16	0.50	0.90	apoptosis
222381_at	programmed cell death 6	PDCD6 or ALG2	1.72	0.92	0.55	1.49	0.69	1.68	2.36	1.29	1.40	apoptosis
204413_at	tnf receptor-associated factor 2	TRAF2	1.01	0.55	0.48	0.70	0.91	1.16	0.55	0.43	0.64	apoptosis:TNF
221571_at	tnf receptor-associated factor 3	TRAF3	1.61	1.64	2.15	1.33	1.75	0.19	0.11	0.03	0.42	apoptosis:TNF
207196_s_at	tnfaip3 interacting protein 1	TNIP1	2.50	2.78	3.48	3.54	2.45	0.13	0.09	0.15	0.12	apoptosis:TNF
206133_at	xiap associated factor-1	XAF1	1.77	0.87	1.02	-0.07	-1.20	-0.44	-1.09	-1.62	-2.76	apoptosis:TNF
202687_s_at	tumor necrosis factor (ligand) superfamily, member 10	TNFSF10 or TRAIL	1.01	0.69	0.40	0.25	-0.78	-0.20	-0.35	-1.36	-3.09	apoptosis:TNF
207113_s_at	tumor necrosis factor (tnf superfamily, member 2)	TNFA	3.61	2.04	2.30	1.12	1.09	-1.32	-0.40	-1.18	-0.69	apoptosis:TNF
209295_at	tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B or TRAILR2	1.99	2.08	2.18	2.27	2.37	0.06	-0.01	0.36	0.46	apoptosis:TNF
204933_s_at	tumor necrosis factor receptor superfamily, member 11b	TNFRSF11B or OPG	0.78	0.70	0.89	0.59	0.58	0.25	0.01	-0.07	-0.11	apoptosis:TNF
207643_s_at	tumor necrosis factor receptor superfamily, member 1a	TNFRSF1A or TNFR1	0.56	0.54	0.70	0.56	0.47	0.05	0.10	0.32	0.57	apoptosis:TNF
218856_at	tumor necrosis factor receptor superfamily, member 21	TNFRSF21 or DR6	1.09	1.30	1.61	1.68	1.80	0.17	0.05	0.97	0.95	apoptosis:TNF
202510_s_at	tumor necrosis factor, alpha-induced protein 2	TNFAIP2	8.67	7.94	8.85	5.94	3.82	0.10	-0.10	-0.08	0.15	apoptosis:TNF
202644_s_at	tumor necrosis factor, alpha-induced protein 3	TNFAIP3 or A20	13.35	13.19	13.30	10.97	9.48	0.18	0.02	0.13	0.19	apoptosis:TNF
218129_s_at	nuclear transcription factor y, beta	NFYB	-0.51	-0.47	-0.42	-0.65	-1.32	-0.21	-0.13	-0.39	-0.32	TF
212457_at	transcription factor binding to ighm enhancer 3	TFE3	1.36	0.75	1.18	0.96	0.83	0.13	0.06	0.03	0.11	TF
204798_at	v-myb myeloblastosis viral oncogene homolog (avian)	MYB	-1.12	-1.90	-1.24	-2.76	-2.66	-0.01	0.00	-0.50	-1.12	TF
201466_s_at	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	0.97	1.29	0.78	1.58	2.32	-0.39	-0.09	1.03	1.69	TF: AP1
36711_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog f (avian)	MAFF	2.18	2.44	2.48	2.72	2.06	0.23	0.34	0.93	0.81	TF: AP1
208961_s_at	core promoter element binding protein	COPEB	0.88	0.94	1.05	1.53	1.63	0.03	-0.07	0.70	0.92	TF: B-cell
205659_at	histone deacetylase 9	HDAC9 or MITR	1.21	0.98	2.36	1.87	3.33	0.44	-0.23	0.00	0.08	TF: chromatin
213667_at	snf2-related cbp activator protein	SRCAP	3.51	3.52	2.60	3.95	3.74	0.16	0.12	0.59	0.64	TF: CREB
202724_s_at	forkhead box o1a (rhabdomyosarcoma)	FOXO1A	2.95	2.27	2.13	2.34	3.18	0.58	0.70	0.73	1.90	TF: forkhead
203543_s_at	basic transcription element binding protein 1	BTEB1	0.77	0.69	1.37	1.23	1.26	0.38	0.00	0.25	0.31	TF: GTF
209905_at	homeo box a9	HOXA9 or HOX1G	-0.50	-0.44	-0.61	-1.40	-2.46	0.06	-0.09	-0.45	-1.16	TF: homeo
206858_s_at	homeo box c6	HOXC6	-0.61	-0.55	-0.88	-1.23	-1.24	-0.21	-0.29	-0.68	-0.79	TF: homeo
203920_at	nuclear receptor subfamily 1, group h, member 3	LXRA	-0.54	-0.41	-0.48	-0.62	-0.70	0.02	-0.03	-0.22	-0.32	TF: NHR
209505_at	nuclear receptor subfamily 2, group f, member 1	NR2F1	-1.35	-1.48	-1.69	-3.02	-3.40	-0.15	-0.40	-0.70	-1.01	TF: NHR
204255_s_at	vitamin d (1,25- dihydroxyvitamin d3) receptor	VDR	1.10	0.78	0.96	0.64	0.52	0.12	-0.02	0.22	0.66	TF: NHR
203985_at	zinc finger protein 212	ZNF212	-0.50	-0.26	-0.19	-0.48	-0.61	-0.10	-0.02	-0.21	-0.34	TF: Zn finger
220250_at	zinc finger protein 286	ZNF286	0.78	1.14	1.51	0.87	1.45	0.66	0.56	0.54	0.48	TF: Zn finger
211778_s_at	zinc finger protein 339	ZNF339	-0.63	-0.69	-0.83	-2.30	-5.10	0.01	-0.25	-1.63	-2.45	TF: Zn finger
218149_s_at	zinc finger protein 395	ZNF395 or HDBP2	-0.61	-0.42	-0.61	-1.42	-1.73	0.12	0.06	-0.13	-0.89	TF: Zn finger
219676_at	zinc finger protein 435	ZNF435	-0.74	-0.63	-0.64	-0.69	-1.22	-0.19	-0.37	-0.45	-0.42	TF: Zn finger
220266_s_at	kruppel-like factor 4 (gut)	KLF4 or GKLF	0.62	0.67	1.12	1.09	1.40	0.29	0.50	1.10	1.74	TF: Zn finger
209457_at	dual specificity phosphatase 5	DUSP5 or HVH3	2.17	2.78	3.20	3.69	3.47	0.51	0.58	1.49	1.74	signaling: Ppase dual specificity
201473_at	jun b proto-oncogene	JUNB	1.17	1.50	1.22	1.54	1.22	0.08	0.12	0.27	0.43	signalling: AP1
203751_x_at	jun d proto-oncogene	JUND	0.59	1.09	1.11	1.16	1.01	0.37	0.70	0.78	1.95	signalling: AP1
207121_s_at	mitogen-activated protein kinase 6	MAPK6 or ERK3	0.66	0.58	0.70	0.51	0.67	0.10	0.05	0.24	0.27	signalling: kinase MAPK
207667_s_at	mitogen-activated protein kinase kinase 3	MAP2K3 or MEK3	0.91	1.13	1.48	1.05	1.39	0.43	0.40	0.48	1.01	signalling: kinase MAPK
214786_at	mitogen-activated protein kinase kinase kinase 1	MAP3K1 or MEKK1	0.87	1.22	1.97	0.49	0.21	0.75	0.26	0.11	0.18	signalling: kinase MAPK
206571_s_at	mitogen-activated protein kinase kinase kinase kinase 4	MAP4K4 or HGK	0.60	0.47	0.76	0.46	0.41	0.15	-0.02	0.18	0.35	signalling: kinase MAPK
219290_x_at	dual adaptor of phosphotyrosine and 3-phosphoinositides	DAPP1 or BAM32	0.80	0.93	0.75	1.44	1.08	-0.16	-0.05	0.20	0.15	signaling: PI3K
218373_at	fused toes homolog (mouse)	FTS or FT1	-0.67	-0.60	-0.71	-1.09	-0.86	0.04	-0.12	-0.40	-0.43	signalling: PI3K
203126_at	inositol(myo)-1(or 4)-monophosphatase 2	IMPA2	-0.69	-0.56	-0.61	-0.37	-0.60	-0.04	-0.12	-0.10	-0.07	signalling: PI3K
212239_at	phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	PIK3R1 or GRB1	-0.64	-0.39	-0.88	-0.97	-0.52	-0.01	-0.15	-0.34	-0.24	signalling: PI3K
204286_s_at	phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1 or NOXA	1.27	1.72	1.87	1.47	0.60	-0.41	-0.13	0.06	-0.01	signalling: PKC target

Table 4. List of TNF-α-induced genes that were sensitive to EGCG (CONTINUED)

208300_at	protein tyrosine phosphatase, receptor type, h	PTPRH	0.78	1.16	1.17	2.44	2.52	0.43	0.18	1.25	1.36	signalling: Ppase PTPase
212099_at	ras homolog gene family, member b	ARHB or RHOB	0.54	0.65	0.77	0.85	1.45	0.21	0.44	0.77	1.38	signalling: small GTPase
219026_s_at	ras protein activator like 2	RASAL2 or NGAP	1.36	0.58	1.30	1.61	0.79	1.46	1.01	0.63	0.81	signalling: small GTPase
205786_s_at	integrin, alpha m (complement component receptor 3, alpha (cd11b)	ITGAM or CD11b	1.96	1.87	2.34	2.24	2.30	0.01	-0.35	-1.45	-0.10	cell adhesion
202351_at	integrin, alpha v (vitronectin receptor, alpha polypeptide, antigen cd51)	ITGAV or CD51	1.32	1.58	1.63	1.87	1.70	-0.05	-0.02	0.18	0.02	cell adhesion
202638_s_at	intercellular adhesion molecule 1 (cd54), human rhinovirus receptor	ICAM1 or CD54	7.79	7.13	7.99	4.04	3.71	0.10	0.00	0.02	0.16	cell adhesion
207194_s_at	intercellular adhesion molecule 4, landsteiner-wiener blood group	ICAM4	3.35	2.84	1.61	0.87	0.63	-0.26	-0.26	-0.02	0.12	cell adhesion
205179_s_at	a disintegrin and metalloproteinase domain 8	ADAM8 or CD156	1.11	2.05	3.27	2.05	1.36	0.53	1.35	1.14	0.64	cell adhesion:ADAM
215243_s_at	gap junction protein, beta 3, 31kda (connexin 31)	GJB3 or CX31	1.12	1.58	1.86	2.23	2.14	0.12	0.15	0.83	1.13	cell adhesion:GPC
218182_s_at	claudin 1	CLDN1	1.00	0.94	0.60	0.27	0.30	-0.09	0.36	-0.16	-0.05	cell adhesion:tight junction
214135_at	claudin 18	CLDN18	0.51	0.07	0.28	6.98	6.69	-0.08	0.18	1.05	1.20	cell adhesion:tight junction
204345_at	collagen, type xvi, alpha 1	COL16A1	0.81	0.38	0.93	1.96	1.02	0.38	0.35	0.26	0.47	ECM
205466_s_at	heparan sulfate (glucosamine) 3-o-sulfotransferase 1	HS3ST1 or 3OST1	0.89	0.81	0.86	1.91	2.60	0.11	0.01	0.53	0.88	ECM
202267_at	laminin, gamma 2, transcript variant 1, mrna	LAMC2	4.39	4.48	5.98	6.87	8.32	0.15	0.26	0.56	0.59	ECM
203726_s_at	laminin, alpha 3	LAMA3	2.12	1.88	1.87	1.79	2.16	0.42	0.22	0.31	0.53	ECM
209270_at	laminin, beta 3	LAMB3	1.29	1.53	1.92	1.78	2.08	0.32	0.15	0.16	0.30	ECM
202071_at	syndecan 4 (amphiglycan, ryudocan)	SDC4	4.65	4.19	3.68	3.49	3.15	0.05	0.05	0.02	0.17	ECM
204259_at	homo sapiens matrix metalloproteinase 7 (mmp7), mrna	MMP7 or PUMP1	2.59	2.68	2.65	4.05	4.34	-0.01	0.09	0.77	0.74	ECM: MMP
208322_s_at	sialyltransferase 4a (beta-galactoside alpha-2,3-sialyltransferase)	SIAT4A	2.00	2.50	2.84	2.79	3.10	0.30	0.34	1.02	1.72	ECM: enzyme
203691_at	protease inhibitor 3, skin-derived (skalp)	PI3 or SKALP	18.84	15.21	8.14	21.80	15.08	-0.91	-0.95	-0.60	-0.46	ECM: protease inhibitor
213112_s_at	sequestosome 1	SQSTM1 or p62	8.74	9.83	10.35	6.52	5.65	1.06	0.06	0.26	1.32	ECM: protease inhibitor
211924_s_at	plasminogen activator, urokinase receptor	PLAUR or CD87	1.42	1.09	1.16	12.59	7.26	-0.19	-0.09	2.72	2.93	ECM: protease; Upar
206404_at	fibroblast growth factor 9 (glia-activating factor)	FGF9	-1.97	-1.64	-1.70	-3.44	-3.83	-0.13	-0.06	-0.72	-1.16	growth factor/receptor:FGF
205266_at	leukemia inhibitory factor (cholinergic differentiation factor)	LIF	2.15	1.83	2.65	3.36	3.80	0.13	-0.22	0.44	2.03	growth factor/receptor:LIF
204200_s_at	platelet-derived growth factor beta polypeptide	PDGFB or PDGF2	4.65	4.20	5.62	6.07	6.81	0.60	0.31	0.82	1.12	growth factor/receptor:PDGF
210355_at	parathyroid hormone-like hormone	PTHLH or PTHR	1.63	2.51	2.72	1.74	3.16	0.83	0.62	0.95	2.19	growth factor/receptor:PTH
221577_x_at	growth differentiation factor 15	GDF15 or MIC1	1.04	0.94	0.49	2.79	1.09	-0.01	-0.31	1.30	0.99	growth factor/receptor:TGFb
205397_x_at	smad, mothers against dpp homolog 3 (drosophila)	SMAD3	4.71	6.75	6.60	5.02	4.38	0.94	-0.12	-0.10	0.46	growth factor/receptor:TGFb signalling
208044_s_at	peroxisome proliferative activated receptor, delta	PPARd	0.52	0.44	0.74	2.63	2.96	0.55	0.54	2.02	2.63	peroxisome
202863_at	nuclear antigen sp100	SP100	0.80	0.65	0.38	0.00	-0.51	-0.01	-0.21	-0.57	-0.86	nucleus
209761_s_at	sp110 nuclear body protein	SP110	0.70	0.23	0.07	-0.33	-0.82	-0.15	-0.37	-0.96	-2.14	nucleus
204279_at	proteasome (prosome, macropain) subunit, beta type, 9	PSMB9 or LMP2	1.76	1.15	0.66	0.44	0.15	-0.40	-0.39	-0.74	-0.48	ubiquitination
205890_s_at	ubiquitin d	UBD	70.48	57.89	49.53	44.46	30.12	0.00	0.00	0.00	0.00	ubiquitination
219211_at	ubiquitin specific protease 18	USP18 or ISG43	1.05	0.53	0.56	-0.23	-0.90	0.01	-0.36	-0.57	-1.24	ubiquitination
201649_at	ubiquitin-conjugating enzyme e2l 6	UBE2L6	0.86	0.32	0.18	-0.11	-0.63	-0.16	-0.39	-1.06	-1.09	ubiquitination
202284_s_at	cyclin-dependent kinase inhibitor 1a (p21, cip1)	CDKN1A or p21	2.52	2.25	2.52	5.72	4.36	0.45	0.69	1.10	1.67	cell cycle, DNA replication&repair
205848_at	growth arrest-specific 2	GAS2	-0.80	-1.24	-0.81	-1.13	-2.07	-0.22	-0.23	-0.29	-0.67	cell cycle, DNA replication&repair
201939_at	polo-like kinase 2 (drosophila)	PLK2	1.84	2.22	2.58	2.74	2.31	0.12	0.19	1.13	1.25	cell cycle, DNA replication&repair
213865_at	discoidin, cub and lccl domain containing 2	DCBLD2	2.06	3.60	5.41	3.40	4.93	0.00	0.00	0.26	3.18	cell cycle: suppressor of cell division
201325_s_at	epithelial membrane protein 1	EMP1 or TMP	0.65	1.65	1.70	4.80	7.08	0.79	0.86	4.31	4.29	cell-cell interaction & control of cell proliferation
203505_at	atp-binding cassette, sub-family a (abc1), member 1	ABCA1	-0.55	-0.32	-0.15	-1.13	-1.22	0.09	0.13	-0.50	-0.46	channel
200045_at	atp-binding cassette, sub-family f (gcn20), member 1	ABCF1	0.50	0.41	0.64	0.33	0.41	0.29	0.21	0.04	0.31	channel
202307_s_at	transporter 1, atp-binding cassette, sub-family b (mdr/tap)	TAP1 or ABCB2	3.89	3.51	3.40	2.20	1.35	-0.07	0.01	-0.19	-0.09	channel
209459_s_at	4-aminobutyrate aminotransferase	ABAT or GABAT	3.26	3.37	3.44	3.78	3.41	-0.59	-0.35	-0.51	-0.49	miscellaneous: enzyme
219181_at	lipase, endothelial	LIPG or EDL	0.90	0.77	0.99	2.35	2.19	0.08	0.22	1.41	1.49	miscellaneous: enzyme_lipid metabolism
202437_s_at	cytochrome p450, family 1, subfamily b, polypeptide 1	CYP1B1	-1.52	-2.16	-2.08	-4.02	-5.77	-0.59	-0.58	-1.16	-1.31	miscellaneous: enzyme_phase I drug metabolism
214235_at	cytochrome p450, family 3, subfamily a, polypeptide 5	CYP3A5	1.08	1.18	1.27	1.95	1.31	-0.05	0.12	0.61	0.39	miscellaneous: enzyme_phase I drug metabolism
205939_at	cytochrome p450, family 3, subfamily a, polypeptide 7	CYP3A7	2.90	3.34	2.43	2.41	2.35	0.80	0.89	0.47	0.39	miscellaneous: enzyme_phase I drug metabolism
204678_s_at	potassium channel, subfamily k, member 1	KCNK1 or TWIK1	0.73	1.25	0.99	2.23	1.72	-0.01	-0.16	0.47	0.39	miscellaneous: ions, vesicles&transport
219593_at	solute carrier family 15, member 3	PTR3	5.38	3.19	3.67	0.00	-0.25	-1.12	-1.12	-1.12	-1.12	miscellaneous: ions, vesicles&transport
221024_s_at	solute carrier family 2 (facilitated glucose transporter), member 10	SLC2A10 or GLUT10	-1.19	-1.00	-0.57	-5.55	-2.12	0.27	0.10	-0.07	-0.51	miscellaneous: ions, vesicles&transport
218725_at	solute carrier family 25 (mitochondrial carrier: glutamate), member 22	SLC25A22 or GC1	1.83	1.92	2.01	1.86	2.04	0.04	0.01	0.13	0.42	miscellaneous: ions, vesicles&transport

Table 4. List of TNF-α-induced genes that were sensitive to EGCG (CONTINUED)

205799_s_at	solute carrier family 3 (cystine, dibasic and neutral amino acid transporters), member 1	SLC3A1 or ATR1	-1.24	-0.75	-0.70	-1.09	-1.33	-0.02	-0.07	-0.32	-1.02	miscellaneous: ions, vesicles&transport
209712_at	solute carrier family 35 (udp-glucuronic acid/udp-n-acetylgalact. dual transporter), member d1	SLC35D1	-0.76	-0.70	-0.87	-1.05	-1.28	-0.12	-0.35	-0.39	-0.58	miscellaneous: ions, vesicles&transport
218928_s_at	solute carrier family 37 (glycerol-3-phosphate transporter), member 1	SLC37A1	1.46	1.51	1.75	1.64	1.82	0.35	0.44	0.70	1.05	miscellaneous: ions, vesicles&transport
209921_at	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	1.39	1.11	1.24	0.63	0.25	-0.27	-0.17	-0.10	0.11	miscellaneous: ions, vesicles&transport
206632_s_at	apolipoprotein b mrna editing enzyme, catalytic polypeptide-like 3b	APOBEC3B	0.51	0.52	0.69	2.28	1.81	0.12	-0.04	0.80	0.86	miscellaneous: lipid transport
209546_s_at	apolipoprotein l, 1	APOL1	0.84	0.69	1.10	1.62	1.10	0.10	-0.02	0.36	0.63	miscellaneous: lipid transport
221087_s_at	apolipoprotein l, 3	APOL3	4.98	3.19	3.43	2.47	1.69	0.84	0.57	1.04	0.49	miscellaneous: lipid transport
205048_s_at	phosphoserine phosphatase	PSPH	-0.87	-0.97	-0.71	-1.17	-1.55	0.20	0.37	-0.02	0.29	miscellaneous
212148_at	pre-b-cell leukemia transcription factor 1	PBX1	-1.24	-1.49	-1.23	-1.65	-3.19	-0.12	-0.06	-0.41	-0.55	miscellaneous
210056_at	rho family gtpase 1	RND1 or RHO6	1.40	2.39	2.19	7.01	3.50	0.54	0.60	1.64	1.09	miscellaneous
221477_s_at	superoxide dismutase 2, mitochondrial	SOD2 or IPO-B	2.09	2.29	2.14	1.01	0.91	0.46	0.47	0.13	-0.06	miscellaneous
217853_at	tensin-like sh2 domain containing 1	TNS1	-0.82	-1.19	-0.60	-1.93	-2.50	0.10	-0.01	-0.53	-0.73	miscellaneous
219274_at	transmembrane 4 superfamily member 12	TM4SF12	-1.01	-0.78	-0.78	-1.89	-2.39	-0.31	-0.05	-0.68	-1.23	miscellaneous
213293_s_at	tripartite motif-containing 22	TRIM22 or STAF50	2.60	1.69	1.77	0.30	-0.07	0.02	-0.03	-0.68	-0.65	miscellaneous
211002_s_at	tripartite motif-containing 29	TRIM29	2.45	0.66	1.70	0.91	1.87	0.55	0.39	0.83	1.08	miscellaneous
215444_s_at	tripartite motif-containing 31	TRIM31	3.02	3.67	3.82	4.09	2.23	0.13	0.09	0.33	0.26	miscellaneous
208178_x_at	triple functional domain (ptprf interacting)	TRIO	1.11	0.86	1.38	1.20	0.93	1.06	0.89	0.63	0.79	miscellaneous
213797_at	viperin	viperin	2.96	1.35	1.88	0.85	0.31	-0.18	0.06	-0.16	-0.44	miscellaneous
206027_at	s100 calcium binding protein a3	S100A3 or S100E	2.93	2.59	2.08	3.21	4.15	-0.55	-0.48	0.25	-0.53	miscellaneous: Ca
205157_s_at	keratin 17	KRT17	2.41	1.71	2.08	3.30	4.46	1.05	1.13	2.80	3.71	miscellaneous: differentiation
214303_x_at	mucin 5, subtypes a and c, tracheobronchial/gastric	MUC5	1.28	1.20	1.96	1.19	0.63	0.22	0.06	0.05	0.25	miscellaneous: differentiation
205064_at	small proline-rich protein 1b (cornifin)	SPRR1B or cornifin	1.17	0.35	0.65	2.00	3.39	0.36	0.05	-0.03	0.09	miscellaneous: differentiation
216336_x_at	metallothionein 1a (functional)	MT1A	-0.66	-1.81	-2.49	-2.62	-4.07	-1.63	-2.17	-3.10	-4.17	miscellaneous: ox defense
213629_x_at	metallothionein 1f (functional)	MT1F	-0.70	-1.79	-2.19	-2.20	-2.05	-1.02	-1.10	-1.92	-1.86	miscellaneous: ox defense
206461_x_at	metallothionein 1h	MT1H	-0.64	-2.07	-3.77	-4.95	-9.57	-3.51	-3.85	-2.84	-5.12	miscellaneous: ox defense
200629_at	tryptophanyl-trna synthetase	WARS	1.28	1.12	1.19	0.88	0.79	0.27	0.14	0.13	0.27	miscellaneous: protein biosynthesis

[a] Change factor compared to unstimulated cells

EGCG interferes with genes involved in immune response (Table 4)

Several genes involved in immune response and inflammation were markedly up-regulated by TNF-α. EGCG down-regulated IL-8, GRO-α, MIP-2α, GRO-γ, IP-10 (CXC chemokines), and MIP-3α (CC chemokine) (Fig. 13), corroborating results described in Chapter 1. With regard to IL-8 and MIP-3α, the effect of EGCG was proven on the gene and on the protein level. Furthermore, other chemokines were affected by EGCG. Fractalkine, a CX3C chemokine, was dose-dependently down-regulated by EGCG while IP-9, a CXC chemokine, was inhibited in each concentration tested (Fig. 14; for TNF-α values see Table 4). These indicate that the effect of EGCG was not restricted to one single chemokine subfamily. Moreover, GCP-2, a CXC chemokine, was not altered by EGCG, suggesting that not all chemokines were affected by EGCG.

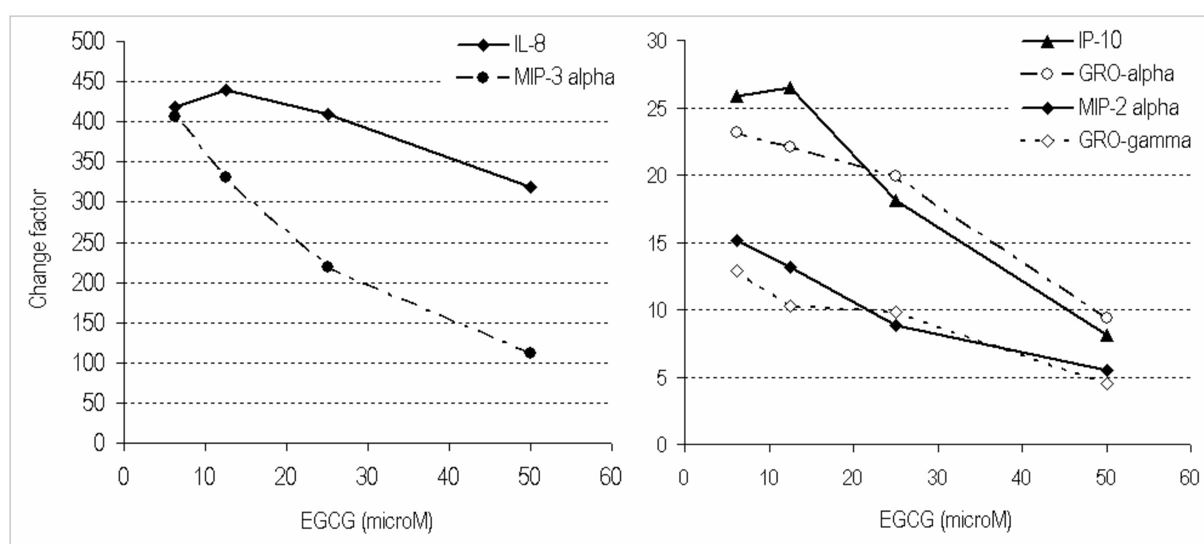


Figure 13: DNA microarray analyses reveal EGCG-modulated chemokine expression

Total RNA was extracted from HT29 cells stimulated with or without TNF-α and exposed to various concentrations of EGCG. cDNA was synthesized followed by hybridization to a microarray chip. Each treatment was done in quadruplicates.

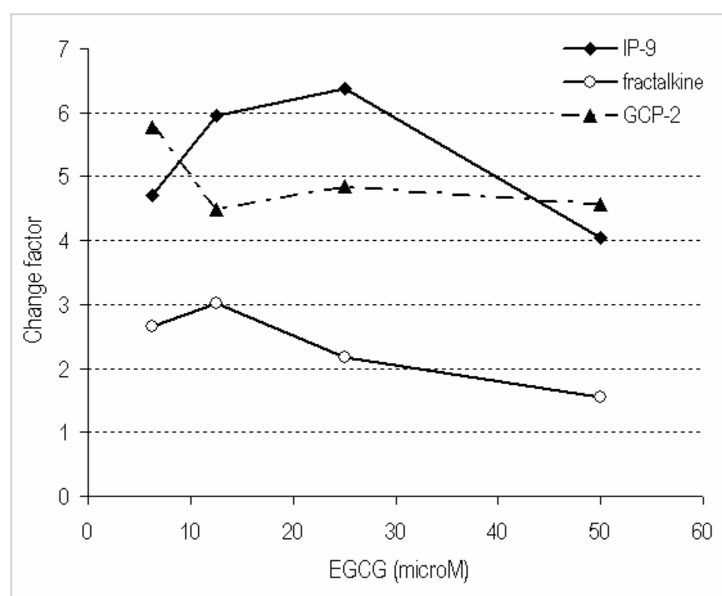


Figure 14: EGCG influenced the expression of chemokines
Dose-dependent down-regulation of IP-9, fractalkine and GCP-2 by EGCG in TNF-α–activated HT29 cells. Each treatment was performed in quadruplicates.

Pro-inflammatory cytokines play an important role in the onset of inflammation (Brynskov et al., 1992). EGCG down-regulated several cytokines which were up-regulated by TNF-α including colony stimulating factor (CSF)-1 and natural killer cell transcription (NK)-4 (also known as IL-32). CSF-1 controls the proliferation and differentiation of macrophages. In addition, it can function as an activator of macrophages and has been implicated as a contributor to disease severity in arthritic animal models (Sweet and Hume, 2003). We suggest that epithelial cells assist macrophages by expressing CSF-1. However, to avoid the activation of a large number of macrophages and to alleviate the severity of the inflammatory process, a limited expression of this cytokine should be present in the mucosa. EGCG reversed the expression of TNF-α-induced CSF-1, controlling macrophage activation and reducing the intensity of the disease. Furthermore, NK-4 was also markedly up-regulated in the presence of TNF-α. Since NK-4 induces the production of TNF-α, IL-8, and MIP-2 in different cell lines (Kim et al., 2005) and the latter cytokines are key molecules responsible for the progression of IBD (Eckmann et al., 1993a; Van Deventer, 1997), only restricted amount of these pro-inflammatory cytokines should be released into the mucosa. EGCG (at 6.25 and 12.5 μM) reduced the expression of NK-4. However, 25 and 50 μM of EGCG slightly up-regulated its expression, suggesting that high concentrations of EGCG rather stimulated NK-4. The regulation of TNF-α, IL-8 and MIP-2 by EGCG is described in Chapter 1.

Interferon (IFN)-γ participates in a large array of cellular responses including the induction of efficient antigen processing for MHC-mediated antigen presentation, which play defined roles in pathogen resistance, and in cell proliferation and apoptosis. Its response is itself regulated by interaction with responses to other cytokines including TNF-α (Boehm et al., 1997). IFN-dependent signalling involves members of four protein families, including the Type I and II IFN receptors (IFNGR); the receptor-associated Janus protein tyrosine kinases (Jaks);

the signal transducers and activators of transcription (STAT) proteins; and members of the interferon regulatory factor (IRF) family of transcription factors (Ramana et al., 2000). Over 200 genes are known to be regulated by IFN-γ, however, only a few were expressed in HT29 cells and up-regulated in the presence of TNF-α (Table 4). EGCG, in at least one concentration, modulated all four members of the IFN signalling. The expression of IFNGR1 and 2, IRF1, and Jak1 were slightly up-regulated in the presence of 12.5 μM of EGCG compared to TNF-α. As deduced by these results, EGCG (at one concentration) was able to activate INF-γ pathway. Furthermore, other molecules belonging to this pathway were also affected. For example, interferon-γ-inducible protein (IFI)-16, a molecule involved in the modulation of p53 and cell proliferation, was dose-dependently down-regulated by EGCG. In the presence of EGCG alone, its expression was further down-regulated. Similar effect were observed on the expression of interferon-induced protein with tetratricopeptide repeats (IFIT)-1 and -3, cellular components of the immune response, where EGCG also reduced their expression. Another interferon-inducible protein, guanylate binding protein (GBP)-1, was increased in cells stimulated with TNF-α. GBP1 is characterized by its ability to specifically bind to the guanine nucleotide GTP, exerting GTPase activities. It is selectively induced by cytokines including IFN-γ and TNF-α, but not by chemokines. It is thought that an elevated expression of GBP1 could inhibit cell proliferation and induce cell adhesiveness for monocytes. In addition, GBP1 was identified as a novel molecular marker of the inflammatory cytokine-activated phenotype of endothelial cells *in vitro* and *in vivo* (Lubeseder-Martellato et al., 2002). In our study, EGCG dose-dependently down-regulated the expression of GBP1. Taking together, these results suggest that EGCG interferes in IFN-γ-induced immune responses, increasing cell proliferation and reducing cell adhesiveness for immune cells.

Binding of a given cytokine (e.g. IFN-γ) to its receptor (e.g. IFNGR) leads to the recruitment and activation of STAT proteins which, after being activated by tyrosine phosphorylation, migrate into the nucleus and mediate transcription of cytokine-specific genes. STATs are key transcription factors which play vital roles in IFN signalling pathway (Ramana et al., 2000). STAT1 is involved in several processes: (1) IκB kinase/NF-κB cascade; (2) STAT protein nuclear translocation; (3) caspase activation which leads to apoptosis; (4) regulation of cell cycle (proliferation); (5) regulation of transcription; and (6) response to pathogen or parasite (Ramana et al., 2000). In our experiment, the expression of STAT1 was up-regulated upon TNF-α stimulation (change factor of 0.66) and significantly reduced by EGCG (50 μM induces a change of -1.05 fold compared to TNF-α). Treating cells only with EGCG also diminished its expression as shown in Table 4. We infer that TNF-α-induced STAT1-signal transducer activities could be reversed in the presence of high concentrations of EGCG.

Moreover, a key enzyme in the biosynthesis of prostaglandins, COX-2, was also expressed in HT29 cells and markedly increased in cells exposed to TNF-α. EGCG dose-dependently reduced its expression, confirming previous results presented in Chapter 1. Lymphotoxin beta (LTB, also known as TNFC), a type II membrane protein of the TNF family, is an inducer of the inflammatory response and involved in the normal development of intestinal lymphoid tissue. Data currently available indicate that the inhibition of LTB receptor signalling leads to the suppression of intestinal inflammation by altering the local intestinal lymphoid microarchitecture (Spahn and Kucharzik, 2004). Our results showed that the expression of LTB was drastically up-regulated upon TNF-α stimulation and EGCG dose-dependently diminished it. Admittedly alteration of the intestinal lymphoid tissue is undesirable, yet EGCG might merely reestablish normal LTB levels, and thus, contribute to the prevention of chronic inflammation.

In summary, EGCG had shown a heterogeneous effect on the expression of genes involved in immune modulation and inflammatory processes in HT29 cells (Fig. 15). It was able to (1) reduce chemokine expression and production, impairing the migration of immune cells; (2) inhibit cytokines such as CSF-1 and NK-4; (3) act on IFN-γ signalling pathway, regulating IFNGR, IRF, JAK1, STAT1 and GBP-1; (4) affect the arachidonic acid metabolism by inhibiting COX-2 and further prostaglandin production; and (5) modulate the intestinal immune system, reducing LTB signalling.

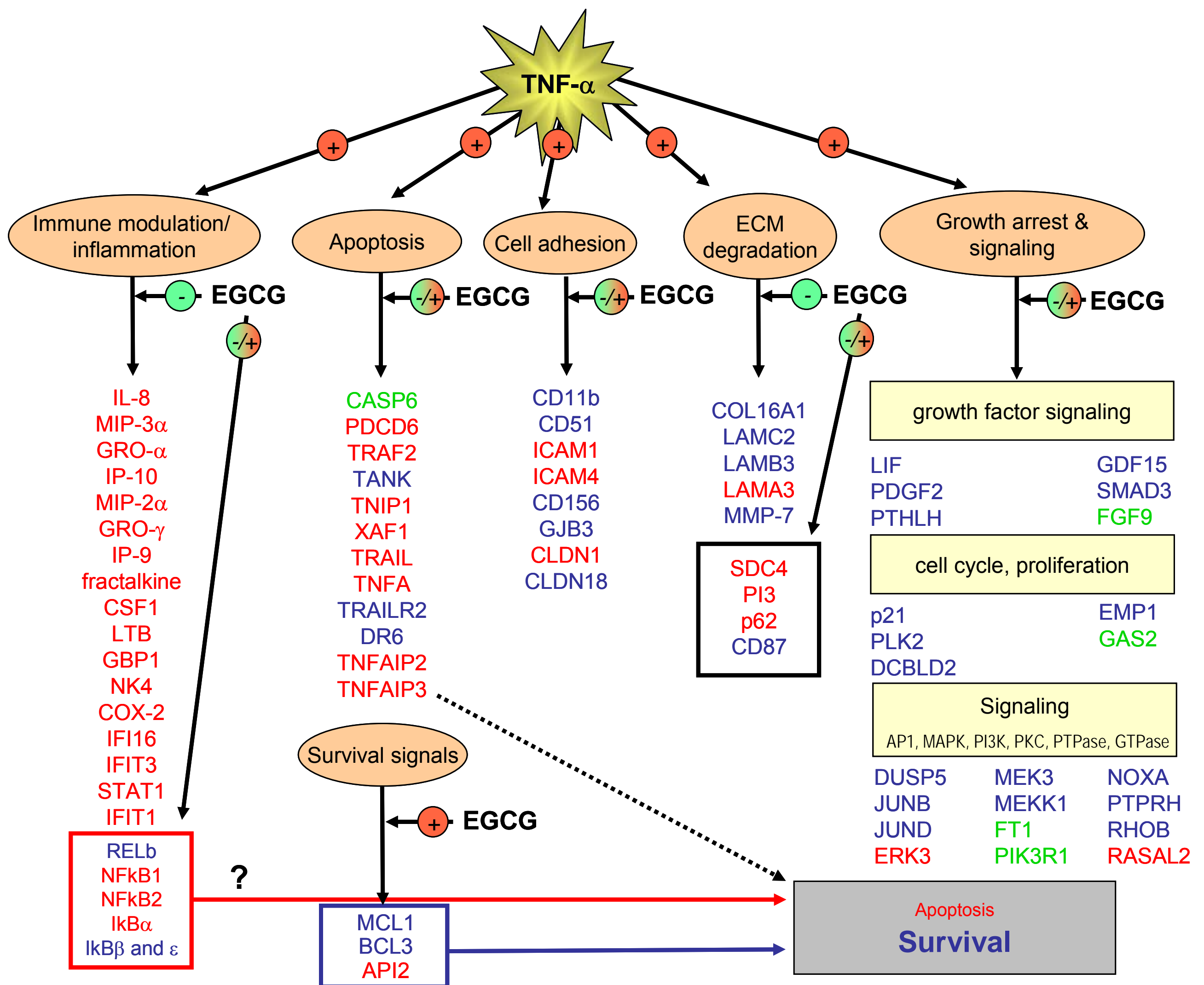


Figure 15: **Effects of EGCG in TNF-α-stimulated HT29 cells**

Model of molecular events, as deduced from the array data. Genes labelled green were down-regulated by TNF-α and subsequently down-regulated by TNF-α / EGCG. Genes labelled blue were up-regulated by TNF-α and further up-regulated by TNF-α / EGCG. Genes labelled red were up-regulated by TNF-α and down-regulated or unaffected by TNF-α / EGCG. For abbreviations, see List of Abbreviations.

NF-κB pathway is modulated by EGCG

NF-κB comprises a family of inducible transcription factors (p50 or NF-κB1, p52 or NF-κB2, p65 or RelA, Rel, and RelB) which is involved in the regulation of important immune processes such as innate immunity, immune surveillance, and antigen presentation. In addition, NF-κB also participates in harmful processes in pathogenesis, *i.e.* in inflammation and cancer (Shishodia and Aggarwal, 2004). It is a heterodimer which usually consists of p65 and p50 (Barnes and Karin, 1997; Yamamoto and Gaynor, 2001). In unstimulated cells, NF-κB is present in a resting state in the cytoplasm associated with the inhibitor of NF-κB (IκB) family (IκBα, IκBβ, IκBε); only when these cells are stimulated (e.g., by cytokines as TNF-α and

IL-1β), NF-κB is activated and translocated into the nucleus where it binds to specific sequences in the promoter regions of target genes to activate gene expression. Several anti-inflammatory substances exert their effects by inhibiting components of the IκB-α/NF-κB cascade (Yamamoto and Gaynor, 2001).

In our experiment, some components of the IκB/NF-κB cascade were induced with TNF-α, including RelB, NF-κB1, NF-κB2, IκBα, IκBβ and IκBε (Table 4). The expression of RelB was slightly up-regulated in the presence of low concentrations of EGCG and reduced with high concentrations. For NF-κB1 or p50, its expression was not altered by EGCG compared to TNF-α. NF-κB2 (p52) expression was drastically up-regulated with low concentrations of EGCG (6.25-25 μM). None of these genes were affected when cells were treated only with EGCG. p65 (RelA) was not found to be regulated by the treatment. It was present in the microarray; however, it possibly was excluded during the filtering due to a low intensity signal or $P > 0.01$. Furthermore, the IκB family were also expressed in HT29 cells upon TNF-α stimulation. IκB-α was 6.76-fold up-regulated in the presence of the stimulus. EGCG inhibited its expression in each concentration tested. IκB-β was only 1.78-fold up-regulated by TNF-α and EGCG rather induced its expression compared to TNF-α. With regard to IκB-ε, its expression was 3.10-fold increased with TNF-α. EGCG up-regulated its expression in each concentration tested. EGCG reversed TNF-α-induced IκB-α expression, while did not affect or rather increased the expression of IκBβ and IκBε, suggesting that it might be able to prevent IκBα degradation.

EGCG regulates apoptosis by modulating TNF-α signalling pathway (Table 4 and Fig. 15)

TNF-α plays an essential role in the activation of caspases and death receptors which lead to apoptosis (Aggarwal, 2003). A dysregulation in the aforementioned process is associated with many pathological conditions such as chronic inflammation and cancer (Zangemeister-Wittke and Simon, 2001).

TNF-induced responses are mediated through one of its receptors, TNFR1 or TNFR2. TNF-α itself is known to drive TNFR1 expression. Upon binding of TNF-α, trimerisation of TNFR1 leads to the recruitment of several proteins to the cytoplasmic “death domain” of the receptor. Association of receptor-interacting protein (RIP) and TNFR-associated factor (TRAF)-2 to TNFR-associated death domain protein (TRADD) activates two transcription factors: NF-κB and activator protein (AP)-1. AP-1-mediated gene induction results from activation of c-Jun N-terminal kinase (JNK) via TRAF2. The transcriptional activity of NF-κB and AP-1 results in suppression of apoptosis. On the other hand, recruitment of fas-associated

death domain protein (FADD) to TRADD allows activation of “initiator” caspases (e.g. caspase 8). The latter indirectly activates the release of apoptogenic mitochondrial factors such as cytochrome c. The association of ATP and the apoptotic protease activating factor (APAF)-1 to cytochrome c leads to the activation of caspase 9 which in turn activates executioner caspases (e.g. caspase 3), leading to apoptosis. Caspase 3 can activate caspase 6, which is involved in the induction of cell shrinkage. Apoptosis inhibitors such as B-cell/lymphoma (Bcl)-2 or Bcl-x_L are able to inhibit the caspase cascade downstream of mitochondria (Papadakis and Targan, 2000; Aggarwal, 2003).

We observed in our experiments that genes of the TNF-signalling pathway were up-regulated with TNF-α, including TNF-α, TNFR1, TRAF2, TRAF family member-associated NF-κB activator (TANK), programmed cell death (PDCD)-6, TNF-related apoptosis-inducing ligand (TRAIL), TRAIL receptor (TRAILR)-2, x-linked inhibitor of apoptosis (xiap) associated factor (XAF)-1, TNF-α-induced protein (TNFAIP)-2 and -3, death receptor (DR)-6, myeloid cell leukaemia sequence (MCL)-1, caspase (CASP)-6, Bcl-3, and apoptosis inhibitor (API)-2. TNF-α expression itself was only 3.61-fold increased after TNF-α stimulation. This contrasts with a substantially higher increase determined by quantitative RT-PCR (see Chapter 1). However, the effect of EGCG on this gene was similar where EGCG dose-dependently down-regulated its expression. In the absence of the stimulus, EGCG down-regulated TNF-α at any concentration. The major effect was observed with the lowest concentration. With regard to TNFR1, its expression was weakly induced upon TNF-α stimulation (0.56-fold). In addition, EGCG was not able to reverse TNF-α-induced gene expression. Overall, the expression level of this receptor in each concentration tested was similar to that of the stimulus or slightly higher. Treating unstimulated cells with 50 μM of EGCG led to an up-regulation of TNFR1 (0.57-fold). Furthermore, the expression of TRAF2, a regulator of NF-κB, was also increased in the presence of TNF-α (1.01-fold). Activated cells treated with EGCG reversed the effect of TNF-α whereas unstimulated cells exposed to EGCG resulted in an up-regulation of this gene. TANK, an inhibitor of TRAF proteins, was induced by TNF-α (0.63-fold). Lower concentrations of EGCG increased its expression. Taken together, EGCG was able to affect TNF-α signalling pathway by inhibiting the expression of TNF-α and modulating its receptor TNFR1. In addition, TRAF2, a regulator of anti-apoptotic signals of TNF receptors, was also reduced by EGCG suggesting that less TRAF2 is available for receptor binding. We deduced that anti-apoptotic signals may be slowed down in the presence of EGCG that thus augments apoptosis. Concomitantly, EGCG up-regulated the inhibitor of TRAF, TANK. However, unstimulated cells treated with EGCG have shown an increase in TRAF2 which could be interpreted as a preventive effect against apoptosis. Since these data

express only effects occurring on the gene level, more experiments need to be done to confirm this hypothesis.

During the normal development of multicellular organisms, cell proliferation, differentiation and cell death are tightly balanced and are regulated by different regulators. Programmed cell death is a genetically controlled process involved in apoptosis and responsible to remove 'unwanted' cells from the organism (Krebs et al., 2002). Programmed cell death (PDCD)-6 was found to be 1.72-fold up-regulated in response to TNF-α in HT29 cells. However, EGCG inhibited it in each concentration tested. Moreover, unstimulated cells exposed to EGCG induced the expression of this gene. These results indicate that EGCG is able to regulate programmed cell death machinery induced in inflammation. Since the epithelium is constantly exposed to various pathogens, immune cells are constantly present in the mucosa. EGCG may increase the expression of cell death proteins in epithelial cell to assist with the normal cellular response, cleaning 'unwanted' cells from the mucosa.

TRAIL, a member of TNF ligand proteins, is involved in apoptosis by binding to and inducing oligomerization of its cell-membrane death receptors (DR4 and DR5), triggering the activity of CASP8 and apoptosis (Guo and Bhalla, 2002). Furthermore, studies demonstrated a relationship between NF-κB and TRAIL (Wajant, 2004). It was reported that NF-κB prevented TRAIL-induced apoptosis in human hepatoma through a TRAIL-activated TRAF2-NIK-IKK pathway (Kim et al., 2002). In our experiment, TNF-α induced the expression of TRAIL (1.01-fold increased) which was dose-dependently down-regulated by EGCG. Exposure of unstimulated cells to EGCG leaded to a subsequent repression of this gene. Moreover, TRAILR2, also known as DR5, was likewise induced by TNF-α (1.99-fold) and further dose-dependently up-regulated by EGCG. No changes on the expression level of this gene were observed after treating unstimulated cells with EGCG. Our findings suggest that EGCG may increase cell surveillance through two ways: (1) direct inhibition of TRAIL-induce apoptosis; and (2) activation of NF-κB cascade by up-regulating RelB and p52. However, the effect of EGCG on TRAILR2 still remains to be addressed. Another gene up-regulated upon TNF-α stimulation was XAF1. This gene antagonizes the anticaspase activity of xiap (Liston et al., 2001) and augments TRAIL-induced apoptosis (Leaman et al., 2002). In activated cells, EGCG had shown an inhibition of the expression of this gene in each tested concentration. In addition, EGCG alone dose-dependently down-regulated it. Furthermore, DR-6, a member of the TNF receptor superfamily, has been shown to activate NF-κB, MAPK8/JNK, and induce cell apoptosis. It has been demonstrated that DR-6 interacts with TRADD activating caspase 8, which in turn activates the NF-κB pathway (Pan et al., 1998). TNF-α induced the expression of DR6 in HT29 cells (1.09-fold increased). In the presence of EGCG, its expression was rather up-regulated compared to TNF-α. Exposure of unstimulated cells to high concentrations

of EGCG also led to an increase of its expression. We infer that EGCG may exerts a preventive effect against apoptosis by affecting TRAIL/DR5 and XAF1 expression, however, not all apoptotic inducers are targets for EGCG.

TNFAIP2, also known as B94, is a cytokine-inducible immediate early gene identified as a TNF-α-inducible transcript in human endothelial cells (Sarma et al., 1992). B94 was shown to be expressed in the intestine (Rusiniak et al., 2000), a finding that is well in agreement with our data presented in this report. We found that TNFAIP2 was moderately up-regulated in the presence of TNF-α (8.67-fold) in HT29 cells. EGCG was able to reduce its expression. Since its function in the intestinal environment is still not elucidated, we were not able to speculate about the effects of EGCG on this gene. Furthermore, another member of TNF-α-inducible protein was found to be expressed in HT29 cells. TNFAIP3, also known as A20, has been implicated in the negative regulation of the NF-κB activation pathway (Lee et al., 2000) and TNF-mediated programmed cell death. It can inhibit TNF-induced apoptosis by disrupting recruitment of death domain-containing adapter proteins TRADD and RIP to the TNF receptor 1 signalling complex (He and Ting, 2002). Upon TNF-α stimulation, the expression of TNFAIP3 was moderately up-regulated (13.35-fold). EGCG inhibited its expression in each tested concentration where the strongest effect was obtained with the highest concentration (50 μM = 9.48-fold). Since the effects of EGCG at low concentrations were not markedly different from that of TNF-α, EGCG did not reverse TNFAIP3 anti-apoptotic effects in these concentrations. However, we can not exclude the possibility that anti-apoptotic proteins may be negatively affected by 50 μM of EGCG.

MCL1, a member of the Bcl2 family, functions as an apoptotic regulator increasing cell survival (Leu et al., 2004). Its expression was 1.23-fold up-regulated in the presence of TNF-α. Treating TNF-α-induced cells with EGCG led to a dose-dependent increase of its expression. Unstimulated cells exposed to EGCG also increased the expression of MCL1, but only at high concentrations (25 and 50 μM). These results indicate that EGCG has a protective effect against apoptosis in both inflammation and normal cellular response. Another anti-apoptotic gene known as Bcl3 was expressed in HT29 cells. Bcl3, a member of the IκB family, was 4.94-fold up-regulated upon TNF-α stimulation. Low concentrations of EGCG increased its expression whereas 50 μM reduced it. Interestingly, unstimulated cells treated with 50 μM of EGCG augmented its expression. These findings suggest that EGCG induces cell survival in inflammatory responses by affecting the NF-κB cascade. Moreover, API2 was shown to be markedly up-regulated by TNF-α (24.30-fold). However, EGCG neither drastically induced nor reduced its expression. Another gene belonging to the caspase cascade, CASP6, was unexpectedly down-regulated with TNF-α, effect of which was intensified by EGCG, indicating that both TNF-α and EGCG would lead to cell surveillance.

Taking together, EGCG had shown heterogeneous effects against apoptosis through the modulation of several genes involved in different pathways. In one hand, regulating genes of the TNF/TNFR1 signalling; and in the other hand, directly affecting death receptors, and thus, the caspase cascade. By regulating TNF/TNFR1 pathway, EGCG inhibited genes involved in the activation of CASP8 in addition to the modulation of others that activated the NF-κB cascade. EGCG also unexpectedly modulated genes that induce apoptosis, indicating that depending of the needs it may induce cell surveillance and apoptosis in epithelial cells.

EGCG regulates cell adhesion in HT29 cells (Table 4 and Fig. 15)

Adhesion molecules regulate the influx of immune cells such as leukocytes in normal and inflamed gut. This process is mediated by adhesion receptor families named integrins, which are particularly important in biological processes that require rapid modulation of adhesion and de-adhesion (Bazan-Socha et al., 2005). In many inflammatory diseases such as IBD, most of the adhesion factors are up-regulated (van Assche and Rutgeerts, 2002). Studies have shown neutrophilic infiltrates within the colonic epithelium of biopsies taken during active diseases periods (Parkos, 1997). Results obtained from our microarray analysis indicated that HT29 cells were able to express some genes involved in cell-cell adhesion, including CD11b, CD51, intracellular adhesion molecule (ICAM)-1, ICAM-4 and CD156. All were up-regulated upon TNF-α activation (1.96; 1.32; 7.79; 3.35; and 1.11, respectively) and regulated by EGCG. The expression of CD11b, CD51, and CD156 (also known as integrin alpha M, V, and a disintegrin and metalloproteinase domain (ADAM)-8 respectively) were up-regulated while ICAM-1 and -4 were down-regulated by EGCG. The latter findings indicate that, in addition to the chemokine inhibition (e.g. IL-8) described in Chapter 1, EGCG can also interfere with leukocyte infiltration by diminishing ICAM, and therefore, ameliorate inflammatory response. We have no worthy explanation for the aforementioned effect of EGCG on CD11b and CD51. The former is mainly expressed in leukocytes and interacts with ICAM-1 upon stimulation. However, we suggest that EGCG would contra-balance its inhibitory effect on ICAM-1 by increasing the expression of CD11b. With regard to CD156, it is expressed in leukocytes and B cells. It interacts with integrins through its disintegrin-like domain, acting as cellular counter receptor (Bridges and Bowditch, 2005). EGCG would increase this interaction, and thus, the function of CD156.

Moreover, gap-junctions and tight-junctions proteins are also involved in cell adhesion. The former are protein-lined channels between adjacent cells that allow the direct cell-cell passage of small cytoplasmic molecules such as ions, metabolic intermediates, and second messengers. The latter are continuous seals (ribbon-like bands) that connect adjacent epithelial cells. They serve as a physical barrier to prevent leakage of fluid across the cell

layer. In our experiment, three genes involved in both mechanisms were found to be up-regulated with TNF-α: gap junction protein beta (GJB)-3; claudin (CLDN)-1 and -18. The expression of GJB-3 was increased in the presence of EGCG. In addition, unstimulated cells exposed to EGCG, also up-regulated this gene, however, only at high concentrations. Furthermore, CLDN-1 was down-regulated by EGCG and its expression was not modulated in unstimulated cells exposed to the compound. The expression level of CLDN-18 was increased when high concentrations of EGCG were used, and it was also elevated in unstimulated cells treated with high concentrations of the substance. We infer that EGCG may affect gap-junctions to increase intercellular metabolic and electronical communication, and thereby, augmenting response to external factors. In addition, increasing the expression of CLDN-18, EGCG would be able to increase tight junction strands in epithelial cells, and therefore, prevent fluid from passing freely through the injured cell layer. Since CLDN-1 was shown to be overexpressed in human colon cancer cell lines (Niwa et al, 2001), we suggest that EGCG may have preventive effects also on colon cancer by reducing CLDN-1.

In summary, our results indicate that EGCG can reduce inflammation by affecting leukocyte infiltration through the reduction of adhesion molecules. In addition, EGCG may regulate cell adhesion by increasing gap- and tight-junction activities between adjacent epithelial cells.

EGCG regulates tissue repair and remodelling (Table 4 and Fig. 15)

Extensive remodelling of the extracellular matrix (ECM) is often found in inflammatory lesions, for example, in IBD. Molecules belonging to the ECM suffer profound alteration and their cell surface receptors have been found in a number of inflammatory processes. In addition, cell-ECM interactions are regulators of these pathological sequences (Smith et al., 1997). Laminins are basement membrane components thought to mediate the attachment, migration and organization of cells into tissues during embryonic development by interacting with other ECM components. In the intestinal tract, laminins influence enterocyte differentiation (Beaulieu, 1997). Laminin (LAM)-5 is an integral part of the anchoring filaments that connect epithelial cells to the underlying basement membrane. It is formed by three subunits (LAMC2; LAMA3; and LAMB3) and beside cell differentiation, it is thought to be involved in cell adhesion and signal transduction (Katayama and Sekiguchi, 2004). We found that LAMC2, LAMA3 and LAMB3 were up-regulated after TNF-α stimulation (4.39; 2.12; and 1.29-fold, respectively). EGCG increased the expression of LAMC2 and LAMB3 in all concentrations tested. The expression of LAMA3 was down-regulated upon EGCG treatment. In addition, EGCG induced the expression of LAMC2 and LAMA3 but not of LAMB3 in unstimulated cells. Furthermore, collagen (COL)16A1, a nonfibril-forming collagen, which serves to maintain the

integrity of the ECM, was also induced upon TNF-α activation (0.81-fold). EGCG augmented its expression when a concentration range of 12.5 to 50 μM was used. EGCG alone did not affect its expression. Moreover, matrix metalloproteinase (MMP)-7, an enzyme implicated in normal and pathologic tissue remodelling processes, was found to be expressed in HT29 cells (Table 4). MMP-7 is involved in wound healing, and studies in mice suggest that it regulates the activity of defensins in intestinal mucosa (Wilson et al., 1999). We observed that the expression of MMP-7 was increased in TNF-α-stimulated cells (2.59-fold). EGCG up-regulated its expression with the increase of the concentration. In addition, its expression was further increased in unstimulated cells exposed to high concentrations of EGCG. Under TNF-α stimulation, the expression of CD87, known as plasminogen activator urokinase receptor (PLAUR), was also up-regulated (1.42 fold). At low concentrations, EGCG rather reduced its expression while at high concentrations it was drastically increased. Additionally, its expression was increased in unstimulated cells treated with high concentrations of EGCG. Since CD87 is involved in the regulation of proteolysis, cell migration and chemotaxis, and growth control; and upon activation, plasminogen augments the degradation of ECM (Wang et al., 2004), we infer that low concentrations of EGCG would rather reduce the activity of CD87, reducing plasminogen-induced ECM degradation. At high concentrations, EGCG would drastically increase the CD87 functions, and thereby, either induce cell migration and chemotaxis, ECM degradation through the activation of plasminogen or even stimulate epithelial cell growth. Our results indicate that EGCG is able to control and reverse ECM degradation induced by TNF-α through the modulation of LAM5 and MMP-7, however, depending on the concentration used, EGCG can also stimulate ECM degradation via plasminogen activation. We suggest that EGCG may promote tissue repair and wound healing by increasing the expression of molecules involved in cell differentiation and tissue remodelling. In addition, EGCG may assist epithelial cells in their function as maintainers of the ECM integrity. Although EGCG, at high concentrations, drastically induced CD87 activity, these effects may contrabalance the inhibitory effect on other genes such as chemoattractant genes, and thus, contributing to physiological functions regulating the quantity of immunocytes present in the mucosa.

In addition to the aforementioned genes, three other genes were found to be indirectly involved in the maintenance of the ECM integrity such as syndecan (SDC)-4, protease inhibitor (PI)-3, and p62 (also known as sequestosome 1). TNF-α induced the expression of all above mentioned genes (4.65; 18.84; and 8.74-fold, respectively). The expression of SDC-4, a transmembrane (type I) heparin sulphate proteoglycan, was dose-dependently reduced by EGCG which did not regulate it in unstimulated cells. Since SDC-4 mediates anti-thrombin-induces chemotaxis of human lymphocytes and monocytes (Kaneider et al., 2002), we imply that EGCG affects inflammatory response by reducing molecules involved in

immunocyte recruitment. Furthermore, EGCG reduced TNF-α-induced PI3, a specific human neutrophil elastase inhibitor. Concentrations of 6.25, 12.5, and 50 μM of EGCG down-regulated PI3 while 25 μM of the substance increased its expression to 21.80-fold. Conversely, a down-regulation of the gene was observed in unstimulated cells treated with low concentrations of EGCG. Since an overexpression of PI3 protected human endothelial cells from neutrophil elastase-induced damage and reduced IL-8 production in response to TNF-α (Henriksen et al., 2004), we infer that only 25 μM of EGCG may induce reparation of damage-tissue and restore TNF-α-induced apoptosis. In addition, EGCG would indirectly interfere with neutrophil migration by increasing PI3 expression and reducing IL-8 (Chapter 1). Moreover, the expression of p62, a gene involved in ubiquitin proteasome degradation, was also modulated by EGCG. Low concentration of EGCG (6.25 and 12.5 μM) rather increased its expression while high concentrations of the compound down-regulated it. Interestingly, only 6.25 and 50 μM of EGCG were able to increase its expression in unstimulated cells. Ubiquitination of cellular proteins is a crucial feature in regulation of signal transduction and cell cycle progression through ubiquitination-dependent proteosomal degradation of important cellular proteins (Vadlamudi et al., 1996). We suggest that EGCG at low concentrations may favour proteosomal degradation while high concentrations would rather inhibit it.

Several signalling pathways are affected by EGCG (Table 4 and Fig. 15)

TNF-α induced the expression of several genes involved in different pathways, including growth factor signalling, cell cycle and proliferation, AP-1, MAPK, PI3K, PKC, PTPase, and GTPase signalling. Firstly, EGCG up-regulated genes associated with growth factor signalling (e.g., LIF, PDGF2, PTHLH, GDF15, and SMAD3) and cell cycle/proliferation (e.g., p21, PLK2, DCBLD2, and EMP1). Secondly, EGCG regulated specific genes of various signalling pathways: AP-1 (JUNB and JUND were increased); MAPK (ERK3 was not significantly affected; MEK3 was increased; MEKK1 was increased with low concentrations of EGCG); PI3K (FT1, PIK3R1 were down-regulated); PKC (NOXA was increased); PTPase (PTPRH was augmented); and GTPase (RHOB was up-regulated; and RASAL2 was inhibited). We concluded that EGCG is able to regulate cell cycle, differentiation, epithelial regeneration, cell growth, in addition to induce wound healing; however, without inducing apoptosis. Since these data are related only to the gene level, further analysis should be elaborated in order to obtain more information about the posttranslational modification such as protein expression.

CONCLUSION

In the present study, we evaluated the effect of EGCG on the expression of an array of genes involved in several processes such as immune response and inflammation, apoptosis, cell adhesion, ECM degradation in addition to its interference in specific signalling pathways. Our findings clearly establish that EGCG influences specific sets of TNF-α-inducible genes resulting in anti-inflammatory effects. Additionally, we provided evidence that EGCG has a heterogeneous effect on these cellular processes. Since this study was performed with only one time point (6 hours), we could not identify genes that changed in expression with time of exposure. Furthermore, microarray analysis deliver a general screening of possible affected genes and indicate potential new leads to mechanisms explaining the biological activity of EGCG. However, data should be confirmed by using other methods, including RT-PCR analysis and protein expression. Moreover, to conclude about the effects of EGCG in a specific pathway, the analysis of genes should be performed as a group rather than a single gene. Overall, studying expression changes of thousands of genes has provided increased insight into the mechanism of action of EGCG in intestinal inflammation, helping us to understand how this compound can be used to prevent or treat inflammatory diseases as IBD.

Chapter 3

Boswellia serrata and *Rosmarinus officinalis* Extracts Impair Chemokine Expression and Production in HT29 Cells

ABSTRACT

Extracts from *Boswellia serrata* (BS) and *Rosmarinus officinalis* (RO) have been used to treat inflammatory conditions, including arthritis and inflammatory bowel disease (IBD). In this study, HT29 cells were used to investigate the effects of BS and RO on activated intestinal epithelial cells. The *in vitro* "inflammatory" condition was induced with tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Activated cells were treated with the indicated extracts to study their effects on cellular inflammatory processes. The secretion of chemokines such as IL-8 and MIP-3α was determined by ELISA. Expression levels of genes involved in inflammation were quantified by real time RT-PCR. NF-κB binding activity was determined by EMSA. IκB-α protein expression was measured by immunoblotting. BS and RO dose-dependently inhibited the synthesis of IL-8 and MIP-3α by activated cells. Concomitantly, BS and RO down-regulated genes involved in inflammatory pathways. NF-κB binding activity was reduced in the presence of RO, but not of BS. The effect of TNF-α on IκB-α was partially reversed by RO while BS had no effect. This study shows the anti-inflammatory effects of BS and RO at different levels in HT29 cells: on the expression of "inflammatory" genes, by affecting NF-κB binding and IκB degradation, and by inhibiting the production of chemokines. Therefore, BS and RO extracts might have a preventive utility in gastro-intestinal disorders and may be further applied in the treatment of inflammatory diseases, including IBD.

INTRODUCTION

The intestinal epithelium forms a physical barrier that protect internal milieu of the host from foreign antigens, eliciting an appropriate immune response. Cells of the gastrointestinal tract, including leukocytes, regulate the immune and inflammatory responses (Kagnoff, 1996; Fiocchi, 1997b). Defects in the mechanisms that control the defence against pathogenic antigens are responsible to initiate an excessive immunological response that may lead to tissue destruction and further intestinal inflammation (Nagler-Anderson, 2001).

During the course of intestinal inflammation, *e.g.* in IBD, epithelial cells develop additional functions which are normally attributed to inflammatory cells. One of these characteristics is that in response to several stimuli, such as TNF-α and IL-1β, epithelial cells initiate an exacerbated production of chemokines which are involved in cell migration (Stenson and MacDermott, 1989; MacDermott, 1994). Chemokines are small chemoattractant molecules responsible in activating and recruiting PML to the injured site (Baggiolini and Moser, 1997; Dwinell et al., 1999). IL-8, MIP-2, GRO-α and GRO-γ belong to the CXC chemokines which mainly attract and activate neutrophils. MIP-3α, a member of the CC chemokine subfamily, is able to activate and recruit monocytes, T lymphocytes and DC. All these molecules are involved in inflammation and contribute to the perpetuation of inflammatory processes (Ajuebor and Swain, 2002).

Beside these molecules, other mediators, including those of the arachidonic acid metabolism, are also relevant in intestinal inflammation. COX-2, the inducible form, is a key-enzyme in the conversion of arachidonic acid to prostaglandins. It is overexpressed in several colon cancers and at sites of inflammation (Parker et al., 1997; Singer et al., 1998). TNF-α and IL-1β are able to induce the expression of this enzyme in a variety of cell lines including the human colon epithelial cells.

Extracts from *Boswellia serrata* Roxb., a large branching tree belonging to the *Burseraceae* family, have been studied in many inflammatory conditions, including arthritic diseases and IBD (Han, 1994; Ammon, 2002). BAs, the major active components present in the gum resin, are responsible for the anti-inflammatory properties of the extract (Ammon et al., 1993; Gupta et al., 1997; Gupta et al., 2001; Krieglstein et al., 2001). Clinical trials in chronic and ulcerative colitis revealed that gum resin preparation from BS improved symptoms of these diseases with minimal side effects (Gupta et al., 1997; Gupta et al., 2001). In a study on Crohn’s disease, BS proved to be as efficient as mesalazine (Gerhardt et al., 2001). Furthermore, in an experimental model of ileitis, the extract of BS and one of its active BAs, dose-dependently decrease rolling and adherence of leukocytes in ileal submucosal venules,

reducing macroscopic and microcirculatory features of inflammation (Krieglstein et al., 2001). The mechanism of action of the extract and its BAs is incompletely understood. Some studies, however, demonstrated that their anti-inflammatory effects were mediated by the inhibition of 5-lipoxygenase (5-LOX) which causes suppression of leukotriene synthesis (Safayhi et al., 1992; Ammon et al., 1993; Safayhi et al., 1995; Sailer et al., 1998).

Rosmarinus officinalis L., a bushy shrub belonging to the family *Labiatae*, has commonly been used to flavour or to preserve food. RO is known for its anti-oxidative (Ozcan, 2003), anti-bacterial (Del Campo et al., 2000), anti-mutagenic (Minnunni et al., 1992), and chemopreventive (Plouzek et al., 1999) properties which are attributed to its active components rosmarinic acid and phenolic diterpenes (e.g. carnosic acid, carnosol, rosmanol). As shown in mouse macrophages, RO exerts anti-inflammatory effects on nitric oxide production through the action of its component, carnosol (Chan et al., 1995). Another study confirmed this finding and revealed that the suppression of nitric oxide synthase was NF-κB-dependent (Lo et al., 2002).

In the present study, we evaluate the anti-inflammatory effects of BS and RO extracts on the TNF-α and/or IL-1β-stimulated human colon adenocarcinoma cell line HT29. Here we show that BS and RO inhibit specific chemokines and modulate several inflammatory genes, suggesting that they can ameliorate intestinal inflammation. Additionally, data presented herein demonstrate that RO, but not BS, reduce the binding activity of NF-κB to its κB element in the promoter sequence, suggesting that it is able to impair the transcription of inflammatory genes.

RESULTS

Evaluation of the impact of plant extracts on cell viability

In order to evaluate the cytotoxicity of the cell treatments and of the extracts, the LDH activity was measured in cell culture supernatants (Fig. 16). EGCG, presented in Chapter 1 and 2, was used as a reference substance. The amount of released LDH was similar in the different treatments, except at 50 μg/mL of BS, indicating that TNF-α/IL-1β and BS or RO did not significantly affect the cell viability.

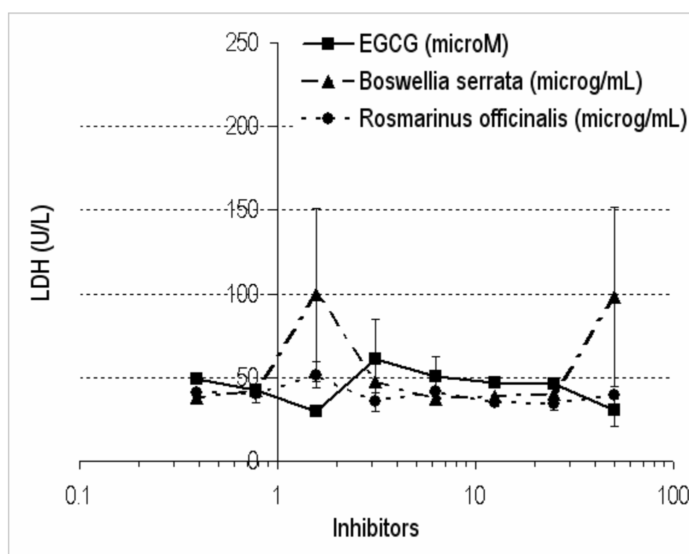


Figure 16: Effects of BS and RO on the LDH release by HT29 cells after 24 h of culture

Cells were stimulated with TNF-α (100 ng/mL) and IL-1β (5 ng/mL) in the presence of varying concentrations of extracts. Amounts of released LDH are expressed in U/L. Similar data have been obtained in two independent sets of experiments.

IL-8 and MIP-3α production is dose-dependently inhibited by BS and RO

IL-8 and MIP-3α production in HT29 cells were measured to investigate the *in vitro* intestinal inflammatory response. Cells responded to TNF-α and/or IL-1β by producing high amounts of IL-8 and MIP-3α (Fig. 17); untreated cells did not secrete detectable amounts of these chemokines. In the presence of IL-1β alone (5 ng/mL), the synthesis of IL-8 was weakly increased compared to TNF-α/IL-1β (Fig. 17A). In cells stimulated only with TNF-α (100 ng/mL), the production of IL-8 was stronger than that observed by IL-1β. The strongest effect was obtained with TNF-α/IL-1β, which drastically raised the production of IL-8 to ~12000 pg/mL.

Furthermore, the effect of the stimuli on the production of MIP-3α was analysed (Fig. 17B). The synthesis of MIP-3α was weakly induced in cells stimulated by IL-1β. In the

presence of TNF-α, the release of MIP-3α was comparable to that observed above. However, this secretion was lower compared to that of IL-8, indicating that the induction of both chemokines is different depending on the stimulus used. Similarly to IL-8, the strongest MIP-3α induction was observed in the presence of TNF-α/IL-1β which possibly reflects a synergistic effect.

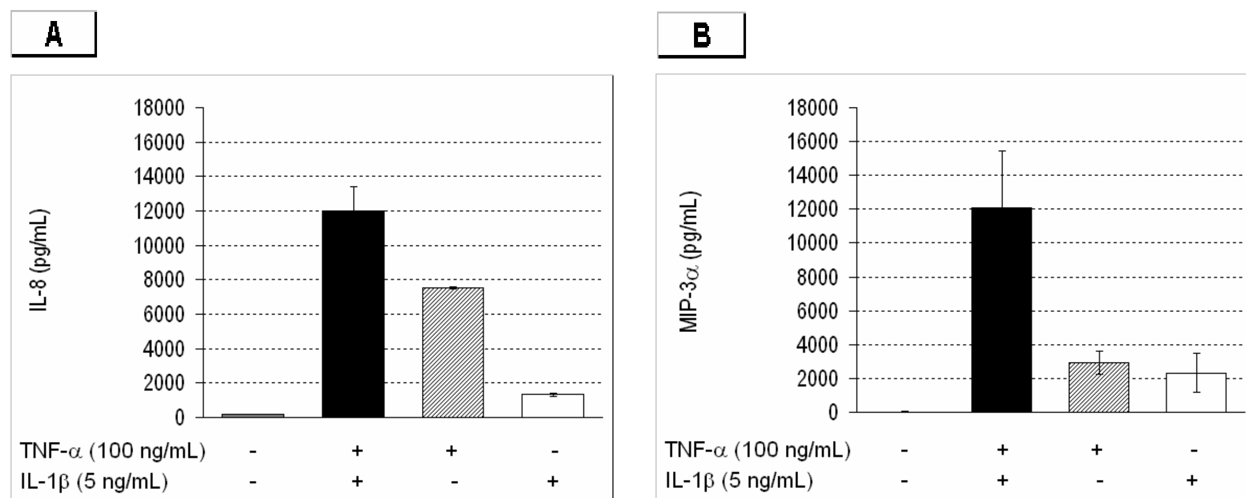


Figure 17: IL-8 (A) and MIP-3α (B) production by unstimulated, TNF-α / IL-1β, TNF-α and IL-1β stimulated cells. Cells were cultured for 24 h and the amount of both chemokines measured in the culture supernatants by ELISA. Results from one of three independent experimental series are shown.

To investigate the effects of extracts on the chemokine production in activated cells, TNF-α/IL-1β-stimulated HT29 cells were incubated with varying concentrations of BS or RO for 24 hours (Fig. 18). These decreased the synthesis of IL-8 in a dose-dependent manner (Fig. 18A). IC_{50} values for BS and RO were 10.0 and 22.6 μg/mL, respectively. Similar results were obtained for MIP-3α, where BS and RO significantly reduced its production (Fig. 18B). IC_{50} values for BS and RO were 2.4 and 3.6 μg/mL, respectively.

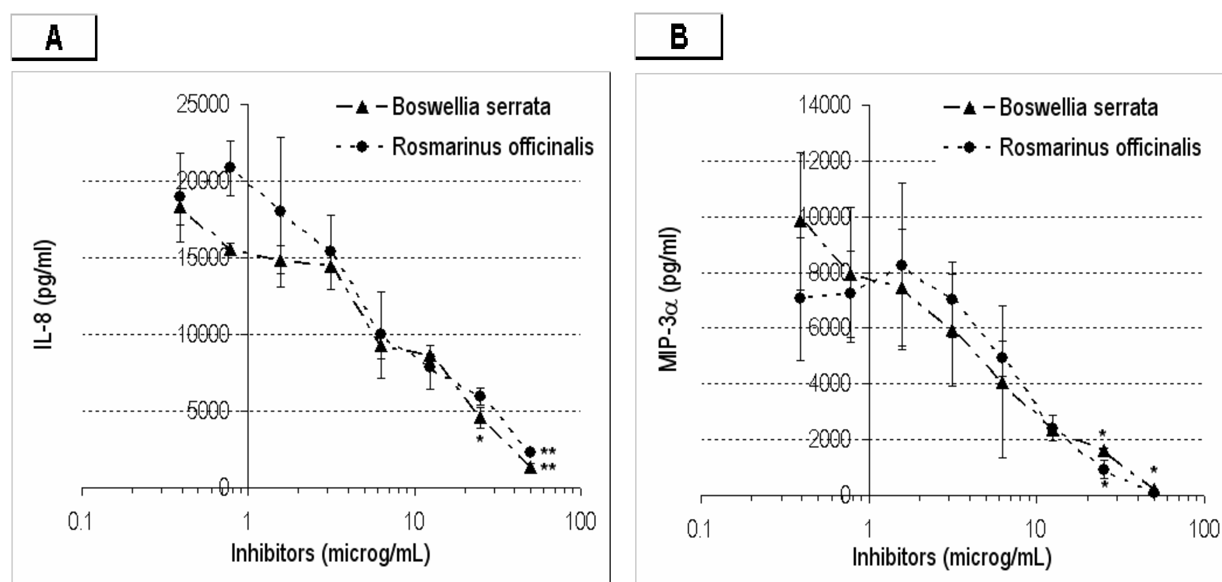


Figure 18: **Effect of BS and RO on the chemokine production**

Levels of IL-8 (A) and MIP-3α (B) in supernatants of TNF-α / IL-1β-stimulated HT29 cells cultured for 24 h in the presence of varying concentrations of the extracts. TNF-α / IL-1β-stimulated cells produced 23697 ± 7292 pg/mL and 12083 ± 3384 pg/mL of IL-8 and MIP-3α, respectively. The secretion of IL-8 and MIP-3α was determined by ELISA. * $p < 0.05$, ** $p < 0.01$. Similar data were obtained in 3 independent experiments.

BS and RO modulate mRNA levels of genes involved in inflammatory responses

During the course of inflammation, a large number of genes are activated. In order to determine these changes, the mRNA levels of unstimulated and TNF-α-stimulated cells were quantified relative to 18S rRNA using the RT-PCR technology. The influence of TNF-α stimulation (Fig. 19) and BS or RO treatment (Fig. 20) was analyzed after 6 hours of culture. TNF-α strongly induced IL-8 and TNF-α; moderately up-regulated MIP-3α, MIP-2, GRO-α, IP-10, GRO-γ; and only weakly modulated COX-2 and RANTES.

Furthermore, the effects of BS and RO were analysed (Fig. 20). TNF-α stimulation resulted in more than 2000-fold increase of the IL-8 mRNA level relative to unstimulated cells. BS and RO diminished this expression, an observation that is consistent with our results of produced IL-8. IC_{50} values for BS and RO were 22.8 and 10.7 µg/mL, respectively, indicating that RO was more potent than BS. With regard to TNF-α, activated cells increased its mRNA levels ~1000-fold. BS and RO reduced it dose-dependently. MIP-3α mRNA levels rose 390-fold after TNF-α stimulation. Both BS and RO impeded it in a dose-dependent way, which is in line with the protein expression. As to MIP-2, an increase of 400-fold in its mRNA was observed after TNF-α stimulation. BS and RO inhibited its expression at each tested concentration. For GRO-α gene, TNF-α stimulation increased its mRNA levels to 220-fold and

BS dose-dependently reduced it. Conversely, RO only modulated it at 50 μg/mL, representing 36% of inhibition. In the case of GRO-γ, activated cells inducted its expression to ~70-fold. It was dose-dependently impaired in the presence of the extracts. IC_{50} values for BS and RO were 21.5 and 11.7 μg/mL, respectively. The expression of IP-10 was also induced by TNF-α stimulation (100-fold increase) and drastically down-regulated by BS. Conversely, RO inhibited it at each tested concentration. Cell activation modestly augmented RANTES mRNA levels (2.4-fold increase); however, both extracts affected its expression. RO revealed an inhibitory effect at all tested concentrations. We also determined the expression of two enzymes involved in inflammatory pathways: COX-2 and 5-LOX. COX-2 was 26-fold increased in the presence of TNF-α. RO dose-dependently reduced its mRNA level (IC_{50} 23.2 μg/mL) while a trend of down-regulation was observed with BS. TNF-α failed to induce the expression of 5-LOX in this cell line.

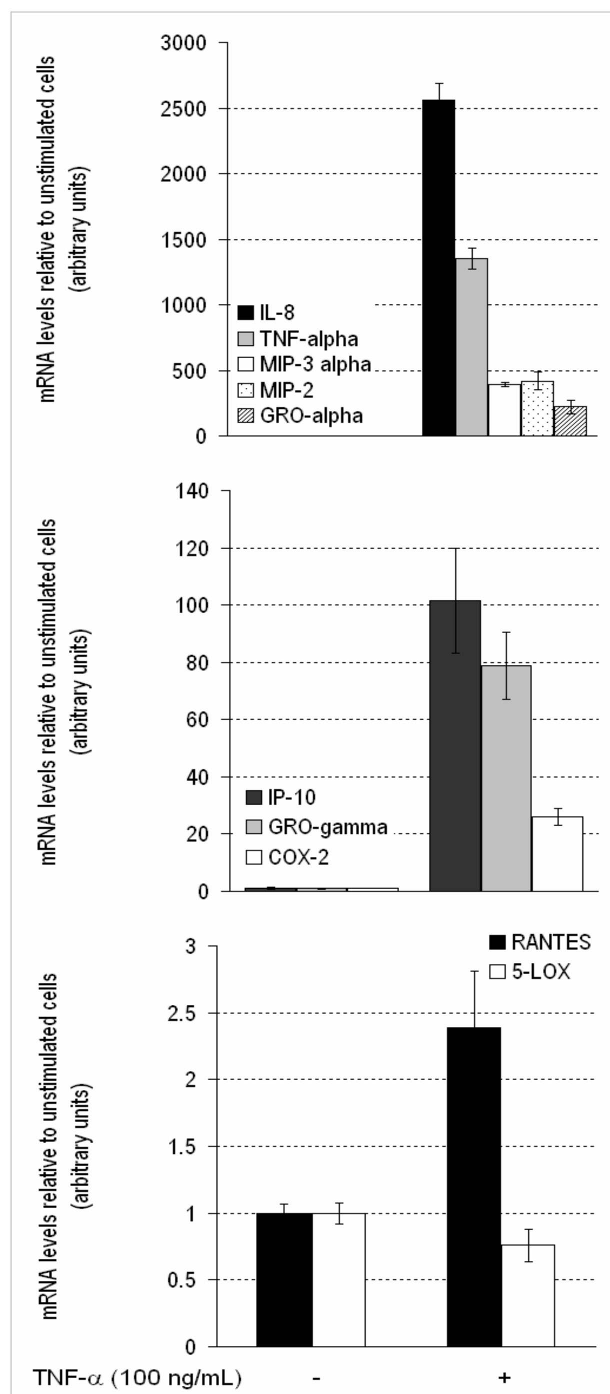


Figure 19: Effect of TNF-α stimulation on gene expression

Cells were cultured without or with TNF-α for 6 h. The expression of several genes was determined by quantitative RT-PCR and the increase of mRNA levels (relative to unstimulated cells) calculated as described in Material and Methods. Representative data of one of two or more independent experiments are shown.

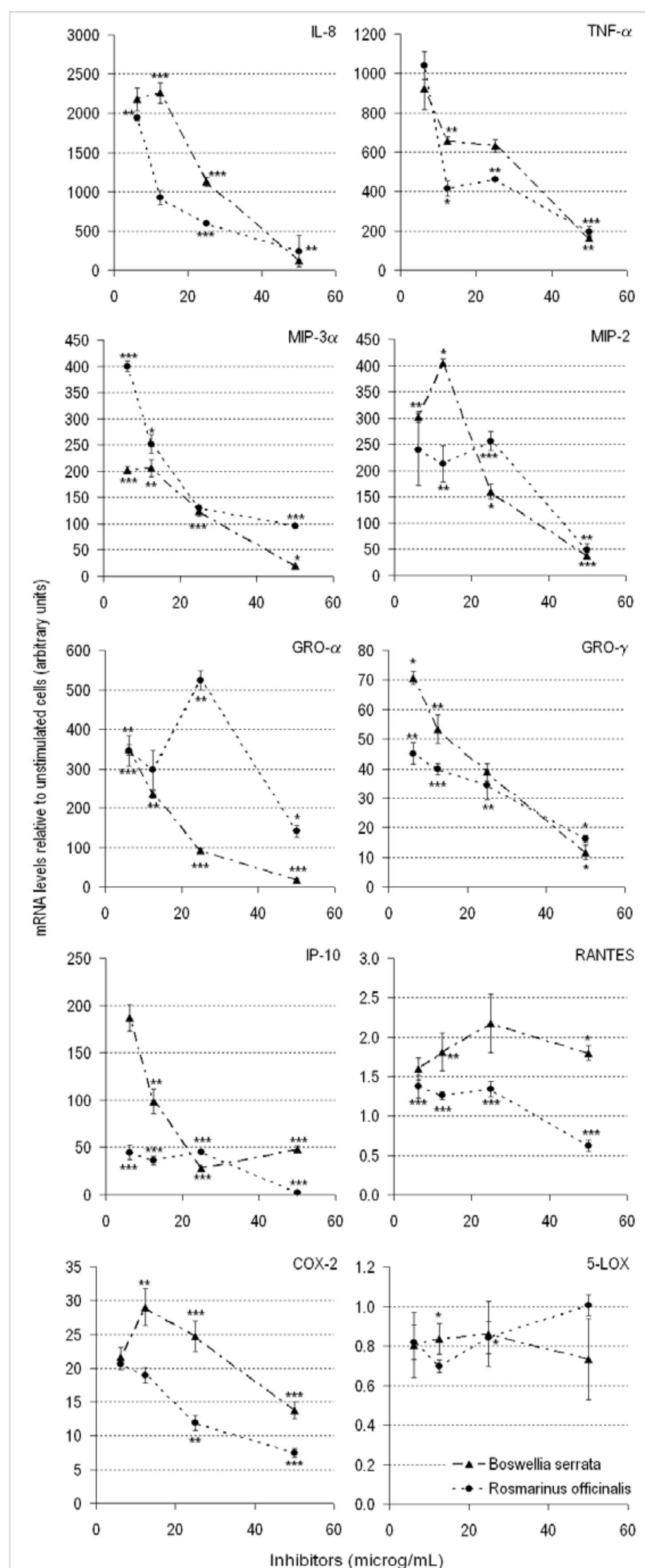


Figure 20: **Dose-dependent effects of BS and RO on several inflammatory genes**
Cells were stimulated with TNF-α for 6 h in the presence of varying concentrations of indicated extracts. mRNA levels were determined relative to unstimulated cells and effects of the extracts compared with those of EGCG (reference compound). See Figure 4 for relative amounts of mRNA in TNF-α-stimulated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to stimulated cells.

BS and RO extracts target NF-κB DNA binding in intestinal epithelial cells

Transcription factors of the NF-κB family regulate the expression of genes involved in inflammation including proinflammatory cytokines (Barnes and Karin, 1997). We investigated the influence of plant extracts on NF-κB activation and its cellular location during *in vitro* inflammation. Nuclear extracts of HT29 cells that were treated with or without TNF-α in the presence or absence of 25 μg/mL of BS or RO were isolated after 10 and 20 min of treatment and analyzed by EMSA (Fig. 21). Treatment of cells with TNF-α induced NF-κB accumulation in the nuclear compartment and thus NF-κB binding activity. BS had no effect on NF-κB binding. After 10 min of incubation, RO slightly diminished (~18 ± 1%, two independent determinations) NF-κB DNA binding (line 6). RO also reduced NF-κB DNA binding after 20 min of incubation (~43 ± 9%; line 12). BS and RO alone did not influence NF-κB DNA binding activity (lines 3-4 and 9-10).

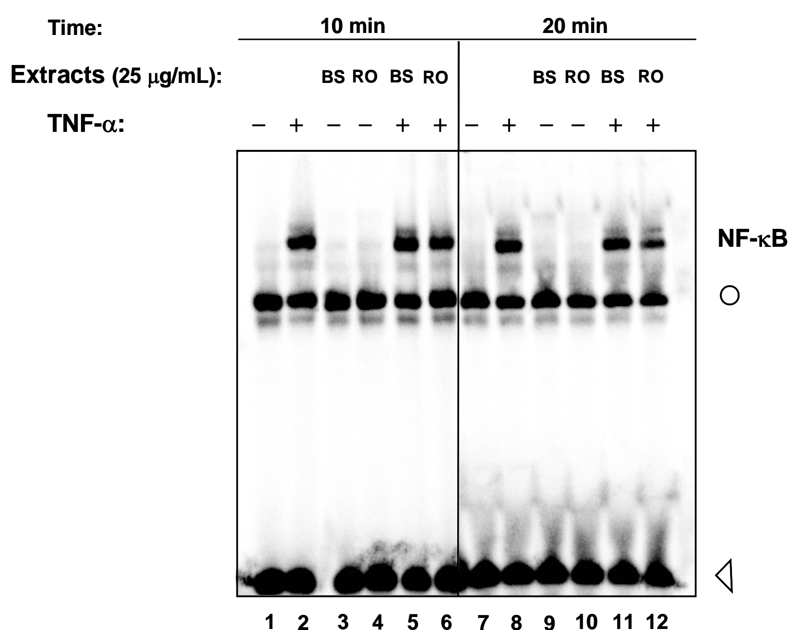


Figure 21: **BS and RO affect NF-κB DNA binding activity in HT29 cells**

Nuclear extracts of unstimulated and stimulated HT29 cells in the presence or absence of BS or RO (25 μg/mL) for 10 (lanes 1 to 6) and 20 min (lanes 7 to 12) were analyzed by EMSA. Lanes 1 and 7: unstimulated cells; lanes 2 and 8: TNF-α stimulated cells; lanes 3 and 9: cells treated with BS; lanes 4 and 10: cells treated with RO; lanes 5 and 11: cells stimulated with TNF-α in the presence of BS; and lanes 6 and 12: activated cells treated with RO. The position of the p50/p65 heterodimer DNA complex is represented in the figure as NF-κB. The open circle denotes a nonspecific activity binding to the probe and the open arrowhead shows unbound oligonucleotide. Results were confirmed in another similar experiment.

Influence of extracts on the IκB-α protein expression

In order to determine the impact of natural extracts on IκB-α protein, cells were stimulated and harvested as described above and the amount of IκB-α determined by immunoblot analysis (Fig. 22). The effects of substances on the NF-κB pathway have been intensively studied in RAW 264.7 cells. For this reason, we used total extracts of this cell line stimulated with or without LPS (1 μg/mL) for 20 min as control. Unstimulated RAW cells had high amounts of IκB-α in their cytoplasm whereas LPS-stimulated cells retained low quantities of this protein, indicating that LPS induces the degradation of IκB-α. In HT29 cells, cytoplasmic extracts expressed high quantities of IκB-α protein depending on the treatment. In contrast, TNF-α-stimulated cells possessed low amounts of this protein, indicating that TNF-α activation resulted in rapid and nearly complete degradation of IκB-α after 10 and 20 min of incubation. Similar results were obtained previously (Elewaut et al., 1999). BS or RO alone increased IκB-α, suggesting that they could prevent IκB degradation. However, treatment of cells with TNF-α in the presence of 25 μg/mL of BS (upper panel Fig. 22) did not reverse the effect of TNF-α after 10 and 20 min. This result suggests that in the presence of a stimulus BS may not prevent IκB-α degradation. Conversely, activated cells treated with 25 μg/mL RO (lower panel Fig. 22) inhibited IκB-α degradation by 43% (after 10 min incubation) and 58% (after 20 min incubation) compared to TNF-α alone (16 and 14%, respectively).

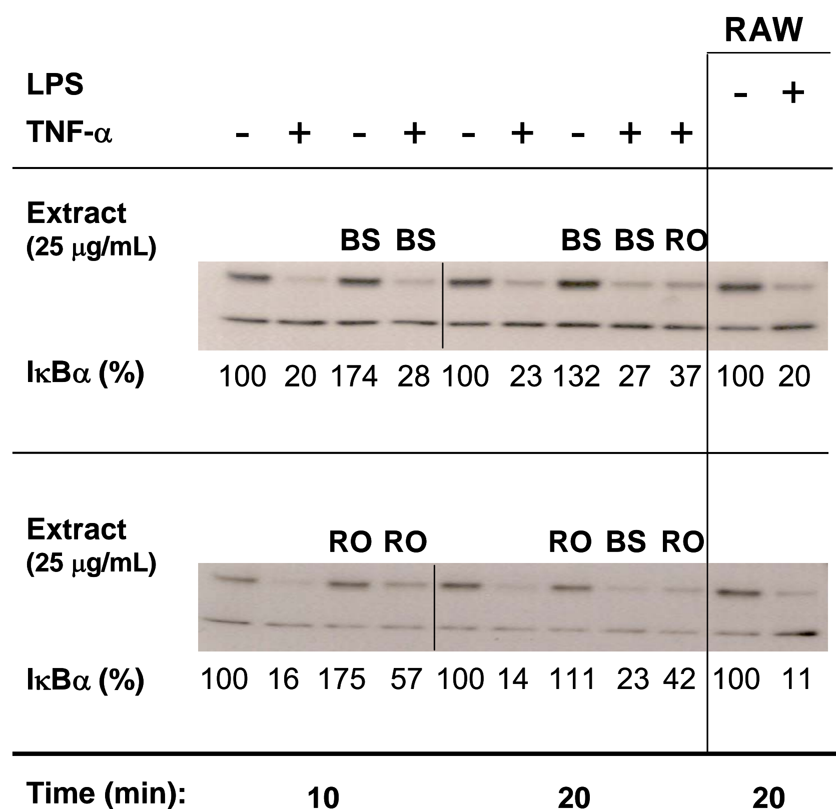


Figure 22: IκB-α protein analysis in cytoplasmic extracts of unstimulated and stimulated HT29 cells

Cells were cultured for 10 and 20 min at indicated conditions and lysates prepared as described in Material and Methods. The effect of BS and RO is shown in the upper and lower panel, respectively. Immunoblot was performed as described above. The blot was further incubated with anti-actin (not shown) and the obtained signal used to normalize for the protein load. Numbers below the lanes indicate the % of IκB-α expressed compared to the amount detected in unstimulated cells. Similar results were obtained in two independent experiments.

DISCUSSION

The aim of this study was to investigate whether natural extracts modulate the *in vitro* chemokine production that occurs during intestinal inflammation. Our experimental approach dealt with the identification of the anti-inflammatory properties of two extracts, *Boswellia serrata* Roxb and *Rosmarinus officinalis* L., in stimulated HT29 cells. The effects of these extracts were determined at three levels: on the expression of genes induced in inflammation, on the NF-κB DNA binding and IκB degradation, and the effect on chemokine synthesis (IL-8 and MIP-3α). BS and RO markedly diminished the production of IL-8 and MIP-3α and the expression of genes involved in the cytokine/chemokine pathways. In addition, RO, but not BS, prevented IκB degradation and reduced NF-κB binding activity. These observations suggest different modes of action of the extracts on cellular metabolism.

In the course of inflammation, the activity of intestinal immune cells are altered, leading to an abundant expression and secretion of cytokines (e.g. TNF-α and IL-1β) and chemokines (Fiocchi, 1997a; Ajuebor and Swain, 2002; Papadakis, 2004). TNF-α and IL-1β are involved in the mediation of the inflammatory response and are found in IBD patients, where they contribute to the status of chronic inflammation (Dionne et al., 1998; D'Haens, 2003). Therefore, these cytokines were chosen to induce an inflammatory state *in vitro*. Once activated, HT29 cells secreted chemokines that are also involved in IBD, IL-8 and MIP-3α. IL-8, together with MIP-2, GRO-α and GRO-γ, is a chemokine belonging to the CXC subfamily and a potent chemoattractant of neutrophils. These chemokines bind to a common receptor CXCR2 (Murphy et al., 2000), and compounds that modulate these chemokines possibly impair biological activities dependent on this receptor. Since neutrophils play a major role in acute mucosal inflammation because of their ability to produce toxic mediators (e.g. reactive oxygen and nitrogen intermediates) in addition to cytokines (Lloyd and Oppenheim, 1992), and due to their vast numbers in inflamed intestine (Podolsky, 1991), an excess of these cells should be avoided in chronic inflammation. In the present study, BS and RO inhibited the production of IL-8 and also down-regulated the expression levels of genes from the CXC subfamily (IL-8, MIP-2, GRO-α and GRO-γ). These findings suggest that BS and RO interfere with the role of these chemokines in attracting CXCR2-bearing inflammatory cells into the inflamed tissue and, therefore, they could ameliorate IBD symptoms.

MIP-3α, a chemokine of the CC subfamily, is highly expressed in human intestinal epithelial cells such as HT29 cells (Izadpanah et al., 2001) and in colonic tissues from IBD patients (Kwon et al., 2002). It is responsible for recruiting mainly DC and memory T cells to the injured area. Since the T cell response is dysregulated in IBD (Fiocchi, 1998), a control of the immigration of these cells is essential to reduce the local inflammation. BS and RO

potently reduced the expression and secretion of MIP-3α in activated cells. Consequently, both extracts could prevent homing of these cells and, therefore, attenuate the inflammatory response.

TNF-α seriously impaired the epithelial barrier function in HT29/B6, a subclone of HT29 cells. This cytokine-induced barrier defect contributes to inflammation due to antigen invasion, and to a loss of ions and water into the intestinal lumen, causing diarrhoea (Schmitz et al., 1999). Activated HT29 cells strongly up-regulated this cytokine and the presence of BS or RO impaired its expression. BS and RO could improve barrier function and interfere with diarrhoea in IBD patients. IP-10, a chemokine belonging to the CXC subfamily, is involved in the recruitment of monocytes and T cells that express CXCR3. In our study, IP-10 was also up-regulated by TNF-α and further influenced by BS and RO, with the latter having a stronger effect. This result suggests that BS and RO are also able to affect activities related to the receptor CXCR3, reducing the Th-1 immune response. RANTES, like MIP-3α, is a CC chemokine and also attracts monocytes, DC and T cells. Upon TNF-α stimulation, RANTES was slightly induced and in the presence of BS and RO, its mRNA level was diminished. With regard to COX-2, a key-enzyme in the synthesis of prostaglandins, it was induced by TNF-α stimulation and inhibited in the presence of BS and RO. COX-2 was reported to be expressed in epithelial cells in areas of inflammation in Crohn's and ulcerative colitis (Singer et al., 1998). This observation explains the large amounts of the prostaglandin E_2 (PGE_2) present in rectal dialysates from patients with ulcerative colitis (Lauritsen et al., 1986). Since PGE_2 induces chloride secretion in epithelial cells, it was suggested that COX-2-related PGE_2 generation contribute to the diarrhoea observed in IBD (Singer et al., 1998). Presumably, the reduction of COX-2 mRNA levels by BS and RO leads to less PGE_2, and therefore, reduction of diarrhoea.

It was shown that the transcription factor NF-κB is involved in the regulation of inflammatory processes (Kumar et al., 2004). Since its activation is associated with chemokine gene expression, the involvement of the NF-κB pathway was analyzed in HT29 cells. In unstimulated cells, NF-κB is retained in the cytoplasm where it is bound by IκB, a complex of inhibitory proteins (e.g. IκB-α and IκB-β), which prevent it from entering the nucleus. When these cells are activated, IκB is phosphorylated and degraded, allowing nuclear translocation of NF-κB (Barnes and Karin, 1997). Our findings indicate that only RO, but not BS, is capable of inhibiting IκB-α degradation (as shown in Figure 22) and consequently NF-κB activation, suggesting that one mechanism of action of RO is through the NF-κB pathway. By preventing IκB degradation, RO impairs the transcription of inflammatory genes and may inhibit early steps of inflammation modulating the regulation of multiple proinflammatory genes.

In conclusion, we showed in this study that extracts of *Rosmarinus officinalis* and *Boswellia serrata* attenuate the inflammatory response in HT29 cells by modulating the expression of multiple genes, regulating NF-κB activation, and inhibiting IL-8 and MIP-3α production. RO is able to interfere with the cytokine/chemokine expression in the NF-κB pathway, binding to NF-κB in the nucleus. With regard to BS, this mode of action could not be applied because BS affects the expression of NF-κB-regulated genes but it has no influence on the activation of NF-κB. Based on these results, BS and RO are good candidates with a large panel of effects to use further in *in vivo* studies of intestinal inflammation. Therefore, we provide further evidence that natural extracts are able to reduce inflammation *in vitro* and, thus, may be applicable for the prevention and/or attenuation of intestinal inflammation.

Chapter 4

Curcumin and resveratrol modulate chemokine production in human colon adenocarcinoma cell lines

ABSTRACT

Curcumin and resveratrol have anti-oxidant and anti-inflammatory effects and seem to interfere with inflammatory processes at different levels. In this study, we investigated the effect of both curcumin and resveratrol on the expression and production of inflammatory mediators such as PGE_2 and chemokines in intestinal epithelial cells. In order to trigger an "inflammatory" state *in vitro*, human colon adenocarcinoma cell lines HT29 and T84 were stimulated with TNF-α (100 ng/ml) and/or IL-1β (5 ng/ml) in the presence or absence of candidate substances. The secretion of IL-8, MIP-3α and PGE_2 was measured by ELISA. mRNA levels were investigated by quantitative RT-PCR. Stimulation of HT29 cells induced the synthesis and secretion of IL-8, MIP-3α and PGE_2 whereas in activated T84 cells only IL-8 and MIP-3α were released. Curcumin dose-dependently inhibited the synthesis of MIP-3α, but not IL-8, in HT29 and T84 cells. Resveratrol did not alter the production of IL-8 in HT29 cells whereas it induced the secretion of MIP-3α. In T84 cells, resveratrol diminished MIP-3α synthesis whereas no effect was observed on the production of IL-8. Moreover, resveratrol and curcumin diminished the generation of PGE_2 in HT29 cells. These results were extended to the analysis of gene expression in HT29 and T84 cells, however, data were not totally corroborated with previous results. Curcumin and resveratrol reduced mRNA levels of IL-8, TNF-α, MIP-3α, MIP-2, GRO-α and COX-2. The expression of GRO-γ was down-regulated only by curcumin in both cell lines. These data reveal that although the synthesis of IL-8 and MIP-3α were differently affected, their expression, in addition to the expression of other chemokines, was regulated by both curcumin and resveratrol.

INTRODUCTION

Intestinal epithelial cells are the first host to come into contact with invading organisms. During bacterial invasion, these cells serve as an early signalling system to adjacent and underlying immunocytes. Activation of epithelial cells by pathogens and/or cytokines, such as TNF-α and IL-1β, leads to induction of multiple inflammatory mediators responsible for the recruitment of inflammatory cells to the injured site (Kagnoff and Eckmann, 1997). In IBD, the pathogenesis involves the interaction of host's genetic constitution, mucosal immunity, and gut microflora. Such interactions may lead to a state of normal - "controlled" - inflammation or to IBD (Sartor, 1997; Fiocchi, 1998; O'Farrelly, 1998; Shanahan, 2001).

Recruitment of immunocytes, mainly neutrophils and lymphocytes, to the intestinal wall is a central event in the pathogenesis of IBD (Madara, 1997; Izadpanah et al., 2001). As described in Chapter 1, chemokines are responsible for the chemoattraction and activation of inflammatory cells (Ajuebor and Swain, 2002). IL-8, GRO-α, GRO-γ and MIP-2 belonging to the CXC subfamily attract mainly neutrophils whereas MIP-3α, a CC chemokine, chemoattract mainly monocytes and lymphocytes. All these molecules are expressed in epithelial cells such as HT29 and T84 cells. COX, a bifunctional enzyme with cyclooxygenase and peroxidase activities (Zhang et al., 1999), is also involved in inflammatory conditions. The cyclooxygenase activity of this enzyme converts arachidonic acid to prostaglandin G_2, which is then reduced to prostaglandin H_2 through its peroxidase activity. PGH_2 is converted into other prostaglandins by a variety of tissue specific enzymes. COX-1 is constitutively expressed in most tissues and generate prostaglandins during the normal physiology, and its expression is not influenced by the stimuli (DeWitt and Smith, 1988). The inducible form COX-2 induces the generation of prostaglandins, including PGE_2, through various stimuli and is involved in normal and pathophysiological conditions (Cronstein, 2002). Its expression is induced in colonic epithelial cells in IBD (Singer et al., 1998).

Curcuminoids, responsible for the yellow color of turmeric, are food substances found in the root of *Curcuma longa* Linn, a member of the ginger family Zingaberaceae. They have a long history of medicinal use in India and Southeast Asia and exhibit a variety of functions, including the anti-inflammatory activity. Curcumin (diferuloylmethane), the major active substance, represents approximately 90% of the total curcuminoids present in turmeric (Chainani-Wu, 2003). Curcumin has been studied as an alternative treatment in gastro-intestinal disorders. In an experimental murine colitis model, curcumin attenuated colonic inflammation and prevented death in trinitrobenzene sulphonic acid (TNBS)-induced colitis (Sugimoto et al., 2002). Similar results were obtained in another study of TNBS-induced colitis (Ukil et al., 2003). In a rat model of colitis, curcumin prevented and cured TNBS-induced inflammation by inhibiting the activity of NF-κB (Jian et al., 2005). In another experimental

model of colitis, curcumin attenuated dinitrobenzene sulfonic acid (DNB)-induced inflammation in mice. Curcumin suppressed the induction of prostaglandin synthesis by inhibiting the expression and activity of COX-2 in gastro-intestinal epithelial cells (Zhang et al., 1999). Moreover, curcumin was reported to specifically inhibit COX-2 but not COX-1 expression in HT29 cells (Goel et al., 2001). It also affected p38 MAPK activity and the NF-κB pathway (Salh et al., 2003). Furthermore, curcumin blocked cytokine-mediated NF-κB activation and inhibited pro-inflammatory gene expression through the suppression of IKK activity (Jobin et al., 1999). Other studies have shown the chemopreventive effects of curcumin in colon cancer (Plummer et al., 1999; Churchill et al., 2000; Ireson et al., 2002; van't Land et al., 2004).

Resveratrol (*trans*-3,4',5-trihydroxystilbene), a polyphenol found in various plants, including grapes, berries and peanuts, has potent anti-proliferative, anti-carcinogenic, anti-oxidant and anti-inflammatory activities. Moderate consumption of red wine which contains resveratrol reduced risk of cardiovascular disease (Soleas et al., 1997; Kopp, 1998). Resveratrol has also shown an inhibitory effect on the growth of *Helicobacter pylori in vitro*, a gram-negative bacterium associated with the development of peptic ulcers and gastric cancer (Mahady and Pendland, 2000). There are a large number of studies demonstrating the inhibitory effect of resveratrol on the transcription of COX-2 (Elmali et al., 2005). This could explain the anti-cancer and anti-inflammatory activity of resveratrol. In colon cancer cell lines such as HT29 cells, resveratrol diminished COX-1 and COX-2 expression (Sovak, 2001). Resveratrol was reported to inhibit not only COX but also other metabolites of arachidonic acid, including thromboxane B_2, 5- and 15-lipoxygenase (Soleas et al., 2001). Moreover, resveratrol was able to induce cell cycle arrest, differentiation and apoptosis in tumor cells without affecting normal cells and thereby inhibiting tumor progression. The mechanisms of action of resveratrol involved in inflammation and cancer include the (1) induction of p53 tumor suppressor by stimulating p53 extracellular-signal-regulated protein kinases (ERKs) and p38 kinase; (2) inhibition of IκB kinase activity by affecting elements of the signalling pathway such as the p38 MAP kinase; (3) inhibition of Src tyrosine kinase and protein kinase C (PKC); (4) cell cycle arrest, mostly cells in the S phase; (5) inhibition of DNA polymerase (Roemer and Mahyar-Roemer, 2002); (6) inhibition of NF-κB-dependent nitric oxide expression and production (Cho et al., 2002).

The present study was undertaken to investigate the anti-inflammatory effects of curcumin and resveratrol in TNF-α-activated human colon cancer cell lines, HT29 and T84. Their ability on reducing intestinal inflammation *in vitro* was examined through their action on chemokines and inflammatory genes. Our findings clearly demonstrated that curcumin and resveratrol have distinct modes of action, operating differently depending on the parameter tested.

RESULTS

Impact of phenolic compounds on cell viability

The cytotoxicity of treatments was determined by measuring the LDH activity in cell culture supernatants (Fig. 23). For HT29 cells, the amount of released LDH in different treatments was ~60 to ~140 U/L, reflecting < 5% of the total LDH of cells. Furthermore, microscopic visualization of cells treated with TNF-α in the presence of high concentrations of curcumin (> 50 μM) revealed a large number of death cells. In addition, curcumim increased LDH activity, indicating that this substance could affect cell viability. Conversely, cells treated with TNF-α in the presence of 50 μM resveratrol did not augment LDH release. With regard to T84 cells, a similar profile was observed (~60 to ~100 U/L of LDH release). However, treating cells with varying concentrations of curcumin and resveratrol did not significantly affect cell viability (Fig. 23B). These findings suggest that high concentrations of curcumin but not resveratrol are able to increase LDH activity and therefore alter cell viability.

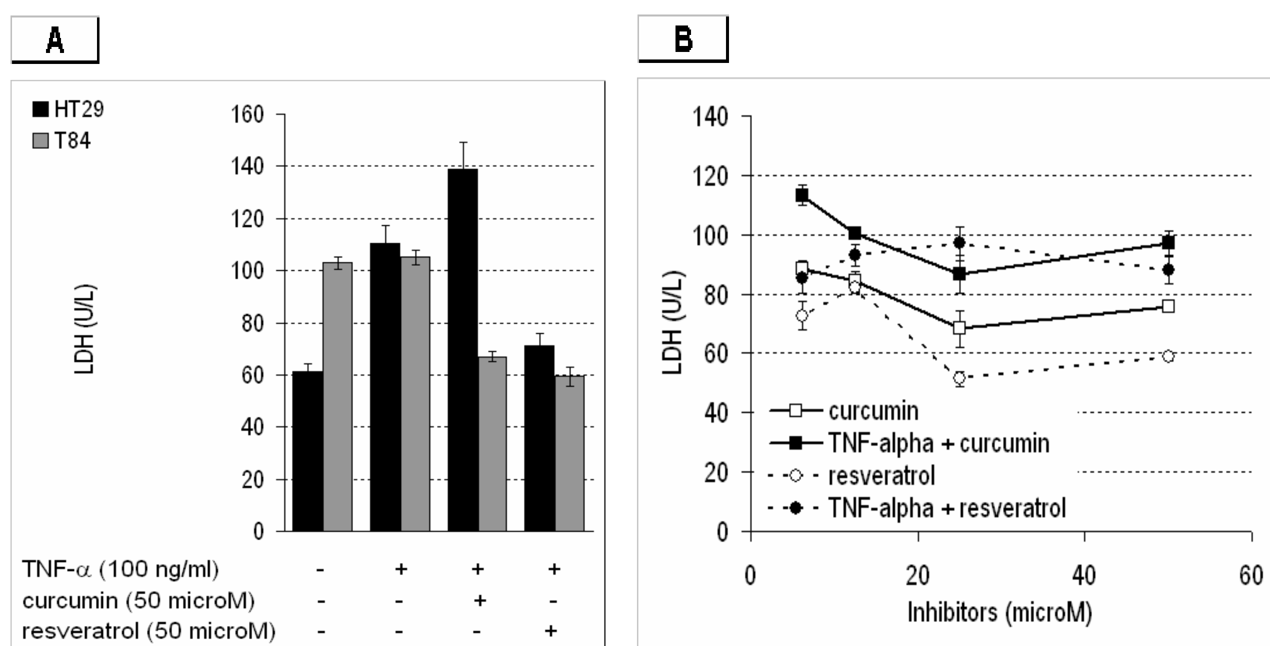


Figure 23: Effects of curcumin and resveratrol on the LDH release by HT29 and T84 cells after 24 hours of culture (A). (B) LDH released in T84 cells with or without TNF-α stimulation in the presence of various concentrations of curcumin or resveratrol. Cells were stimulated with TNF-α (100 ng/mL) in the presence of indicated compunds. Amounts of released LDH are expressed in U/L. Similar data was obtained in two independent sets of experiments.

Impact of phenolic compounds on IL-8 and MIP-3α secretion

In order to measure the effect of curcumin and resveratrol on inflammatory parameters, cells were stimulated with TNF-α in the presence of varying concentrations of substances for 10 hours (IL-8 produced after 10 hours was comparible to 24 hours) and the amount of released IL-8 determined by ELISA (Fig. 24). Activated HT29 cells produced 15429 ± 1088 pg/ml of IL-8 and the presence of curcumin or resveratrol did not alter its synthesis (Fig. 24A). A similar profile was obtained with T84 cells where cell stimulation increased the production of IL-8 to 15376 ± 449 pg/mL, and neither curcumin nor resveratrol reduced it (Fig. 24B).

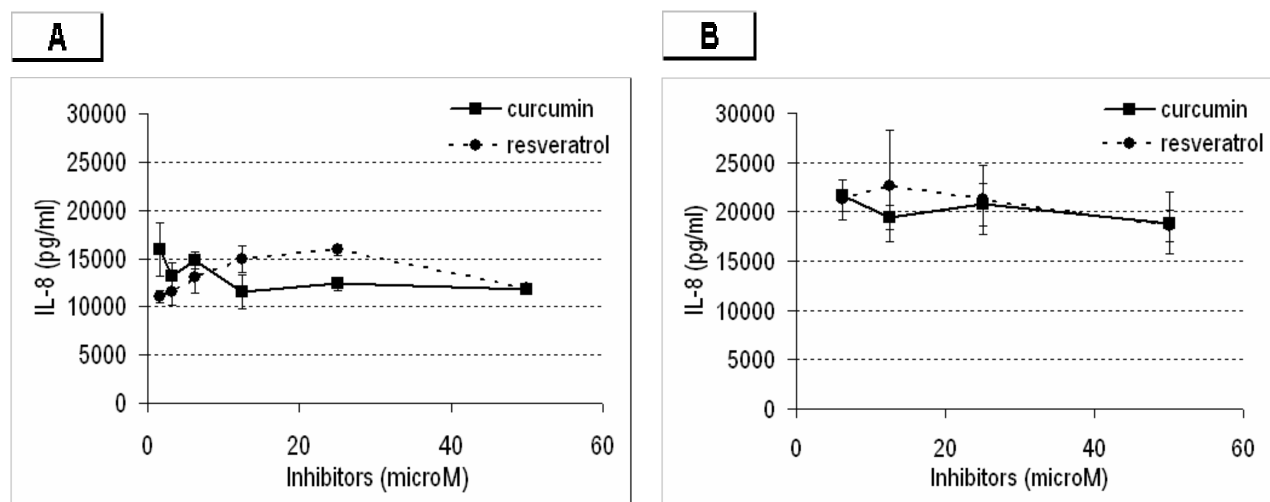


Figure 24: Influence of various concentrations of curcumin and resveratrol on the production of IL-8 by TNF-α / IL-1β-stimulated HT29 (A) and T84 (B) cells

Cells were cultured for 10 hours and the amount of IL-8 in culture supernatants measured by ELISA. Results from one of three independent experimental series are shown.

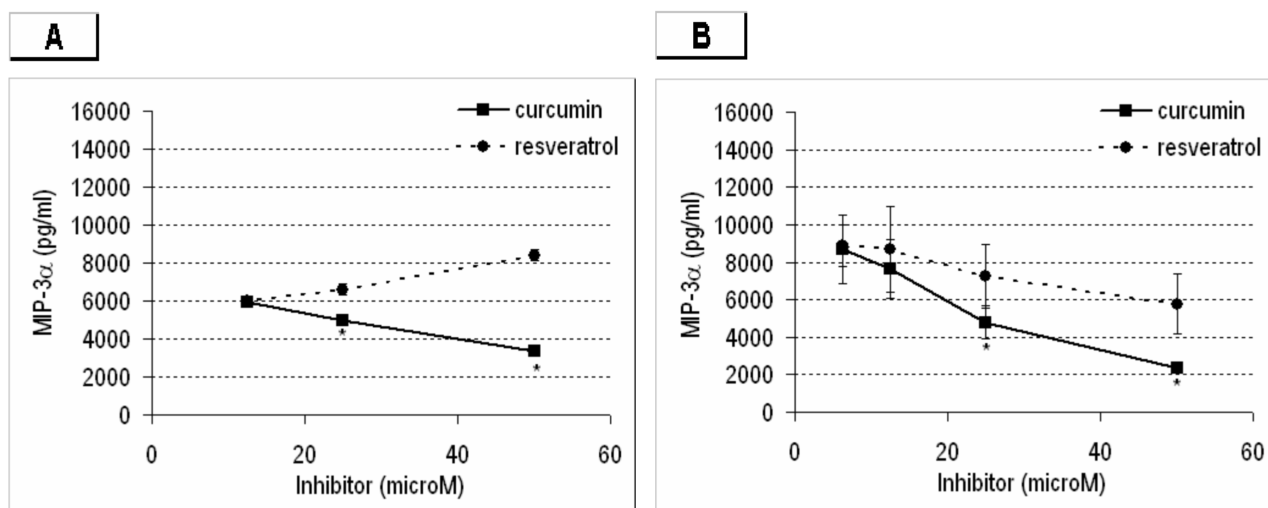


Figure 25: Effect of curcumin and resveratrol on the production of MIP-3α in HT29 (A) and T84 (B) cells
Cells were stimulated with TNF-α in the presence of varying concentrations of candidate compounds. Culture supernatants were obtained after 24 hours and the amount of MIP-3α measured by ELISA. * $p < 0.05$. Representative data of two independent experiments are shown.

Upon stimulation, HT29 and T84 cells also secreted large amounts of the chemokine MIP-3α (8815 ± 628 pg/mL and 10713 ± 629 pg/mL, respectively, as shown in Figure 25). In HT29 cells, MIP-3α production was unaltered by resveratrol, whereas curcumin dose-dependently inhibited its production (Fig. 25A). Resveratrol moderately impaired the amount of secreted MIP-3α while curcumin dose-dependently reduced it in T84 cells (Fig. 25B). IC_{50} values for curcumin and resveratrol were 10.5 and > 50 µM, respectively.

PGE_2 generation is affected by resveratrol and curcumin

The production of PGE_2 by HT29 cells was measured to investigate whether phenolic compounds affect the *in vitro* inflammatory response *via* the arachidonic acid metabolism. To this aim, cells were stimulated with TNF-α for 24 hours and the secreted PGE_2 was determined by ELISA. TNF-α increased the synthesis of PGE_2 to 420 ± 40 pg/mL compared to unstimulated cells (198 ± 30 pg/mL). These were suppressed by resveratrol in a dose-dependent manner and inhibited by curcumin at each tested concentration, indicating that resveratrol was more potent that curcumin (Fig. 26). Under the chosen experimental conditions, T84 did not produce detectable amounts of PGE_2.

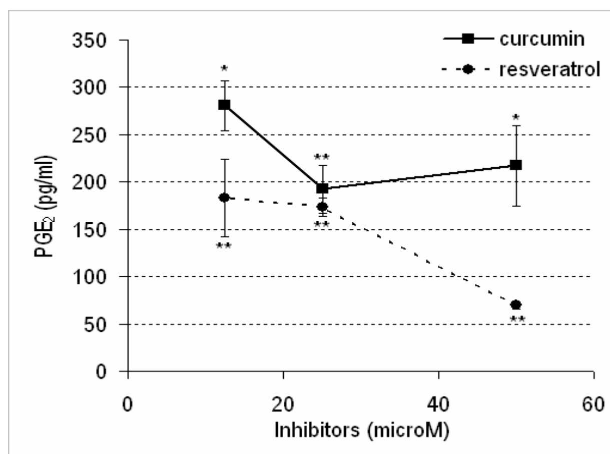


Figure 26: Curcumin and resveratrol inhibited the secretion of PGE_2 in TNF-α-activated HT29 cells

Cells were stimulated with TNF-α (100 ng/mL) in the presence of varying concentrations of candidate compounds for 24 hours. The production of PGE_2 was determined by ELISA. * $p < 0.05$; ** $p < 0.01$. Similar results were obtained in three other series of experiments.

Phenolic substances alter the expression of inflammatory genes in colon cancer cells

Quantitative reverse-transcription polymerase chain reaction analysis showed increased mRNA levels for inflammatory genes, such as IL-8, TNF-α, MIP-3α, MIP-2, GRO-α, GRO-γ and COX-2 in TNF-α-activated HT29 cells after 6 hours (Fig. 27). For IL-8 gene, TNF-α stimulation resulted in > 780-fold increase of mRNA relative to unstimulated cells. Fifty µM of curcumin down-regulated its expression while resveratrol had a weak effect on it. As to TNF-α, stimulation of cells with TNF-α enhanced its expression (220-fold increase). Both curcumin and resveratrol reduced it, however, the inhibition was more marked by curcumin. MIP-3α mRNA levels rose by TNF-α stimulation (280-fold increase). Curcumin and resveratrol diminished it, but the effect of curcumin was more accentuated compared to resveratrol's effect. For MIP-2 gene, we observed a 92-fold mRNA increase by TNF-α stimulation. Curcumin and resveratrol down-regulated the expression of MIP-2. GRO-α mRNA levels were also increased in activated cells (240-fold). Curcumin and resveratrol significantly down-regulated its expression. In the case of COX-2 mRNA, TNF-α stimulation resulted in an up-regulation of the gene (~ 17-fold increase). The addition of curcumin or resveratrol inhibited its expression. For GRO-γ gene, activated cells enhanced its mRNA levels (~ 30-fold increase). Curcumin diminished its expression, whereas resveratrol had no major effect.

Similar profiles were observed in T84 cells, where TNF-α-activated cells increased the expression levels of IL-8, TNF-α, MIP-3α, MIP-2, GRO-α, COX-2 and GRO-γ after 6 hours of culture (Fig. 28). For IL-8 gene, TNF-α stimulation enhanced its expression 1140-fold.

Curcumin and resveratrol (at 50 μM) significantly reduced IL-8 mRNA. These findings were not consistent to the observation made for IL-8 production. TNF-α mRNA levels also augmented by TNF-α stimulation (400-fold increase). Curcumin and resveratrol inhibited its expression. With regard to MIP-3α, an up-regulation of its expression was observed after TNF-α stimulation (~250-fold increase). Both curcumin and resveratrol reduced its mRNA level; however, the inhibitory effect of curcumin was more accentuated. The expression of MIP-2 was likewise elevated with TNF-α stimulation (260-fold increase) and similar to data presented for HT29 cells, curcumin and resveratrol significantly inhibited its mRNA levels. GRO-α gene was 192-fold increased in the presence of TNF-α. Both compounds diminished its expression, but curcumin had a stronger effect. In the case of COX-2 gene, TNF-α stimulation resulted in approximately 10-fold increase of its mRNA relative to unstimulated cells. Curcumin and resveratrol significantly inhibited its expression. GRO-γ mRNA levels rose to 35-fold increase by TNF-α stimulation. Curcumin was able to reduce it whereas resveratrol had no effect on its expression.

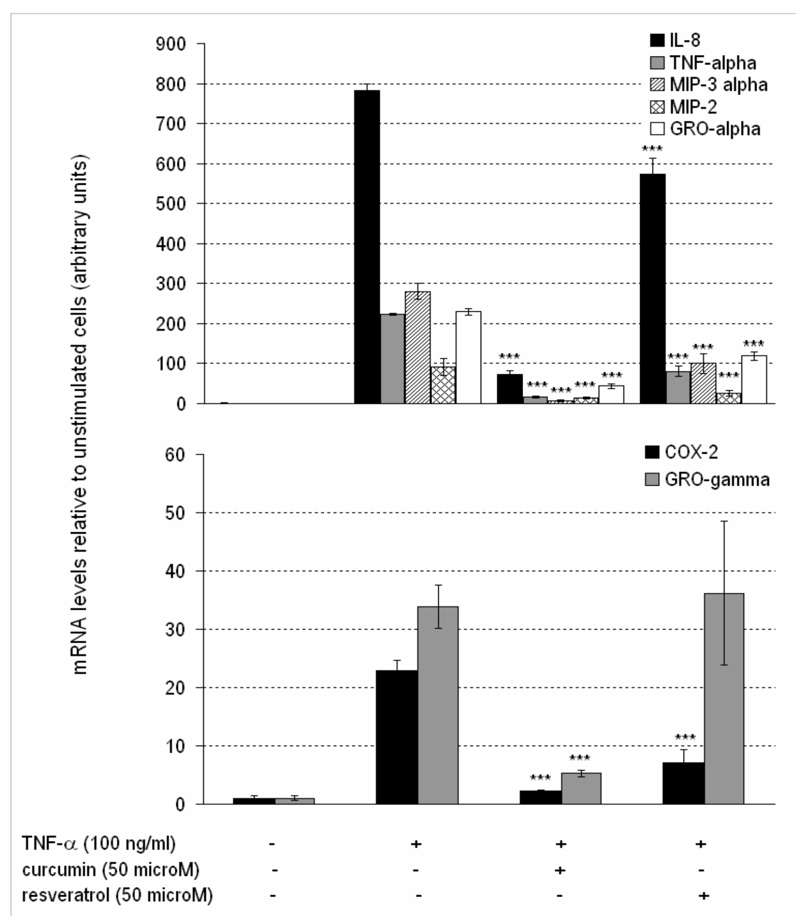


Figure 27: Curcumin and resveratrol modulate genes involved in inflammatory responses

Activated HT29 cells were cultured in the presence of 50 μM of curcumin and resveratrol for 6 hours. The effects of candidate substances on mRNA expression levels of IL-8, TNF-α, MIP-3α, MIP-2, GRO-α, GRO-γ and COX-2 were analyzed by quantitative RT-PCR. *** $p < 0.001$. Representative data of two independent experiments are shown.

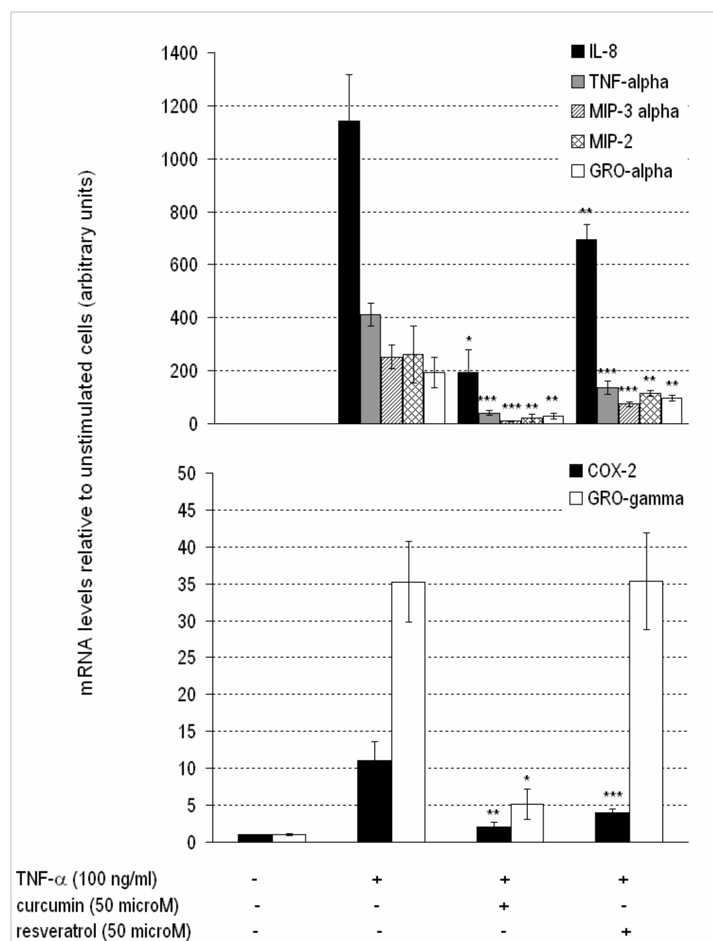


Figure 28: Effect of curcumin and resveratrol on mRNA levels of genes involved in inflammation

T84 cells were stimulated with TNF-α (100 ng/mL) in the presence of 50 μM of candidate substances and cultured for 6 hours. The expression of several genes was determined by quantitative RT-PCR and the increase of mRNA levels (relative to unstimulated cells) calculated as described in Material and Methods. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Representative data of two independent experiments are given.

The effect of various concentrations of curcumin and resveratrol was examined on mRNA levels. In HT29 cells (Fig. 29), TNF-α-induced IL-8 mRNA resulted in > 400-fold increase relative to unstimulated cells. Consistent with the RT-PCR data presented above and conversely to the protein production data, curcumin inhibited IL-8 mRNA levels only at 50 μM while resveratrol reduced it at all tested concentration. For TNF-α gene, we observed an increase of 113-fold on its mRNA levels in activated cells. Although a trend of down-regulation was observed, curcumin diminished TNF-α expression at 50 μM while resveratrol did not affect it. As to MIP-3α, activated cells enhanced its expression (101-fold increase). Curcumin reduced it dose-dependently and resveratrol had no major effect on its mRNA levels. MIP-2 mRNA levels rose by TNF-α stimulation to 104-fold. Curcumin inhibited it in a dose-dependent way. Conversely, resveratrol modulated MIP-2 expression at low, and a trend of up-regulation was observed at high concentrations. For GRO-α gene, activated cells induced its expression to 67-fold. Curcumin dose-dependently diminished its mRNA levels while resveratrol had no effect; however, a trend of down-regulation was observed. In the case of COX-2, stimulation of

cells with TNF-α resulted in a 10-fold increase of the gene. Curcumin and resveratrol had only an effect at high and low concentrations, respectively.

Similar results were obtained with stimulated T84 cells which also up-regulated inflammatory genes. The effect of various concentrations of curcumin and resveratrol were analysed after 6 hours culture (Fig. 30). TNF-α/LPS stimulated cells increased the expression level of IL-8 mRNA to 936-fold. Curcumin had an inhibitory effect only at high concentrations, while resveratrol reduced it at low concentrations. TNF-α mRNA levels drastically rose to 2419-fold by TNF-α/LPS stimulation. Curcumin dose-dependently inhibited its expression. Resveratrol modulated TNF-α mRNA levels in a concentration range up to 25 μM while at 50 μM, it significantly up-regulated its expression. For MIP-3α gene, activation of cells resulted in a 146-fold increase in its mRNA levels. Conversely to the protein data presented above, curcumin significantly affected MIP-3α expression only at 50 μM and resveratrol reduced it at low concentrations. MIP-2 mRNA levels were augmented in the presence of TNF-α/LPS to 250-fold. Curcumin diminished its expression at each tested concentration while resveratrol significantly reduced it at low, and increased it at high concentrations. Stimulated cells induced the expression of GRO-α (184-fold increase) where curcumin modulated it by increasing its concentration. Conversely, resveratrol potentially inhibited its expression at low, and slightly lowered it at high concentration. With regard to COX-2 gene, TNF-α stimulation increased it mRNA levels to 17-fold. Curcumin failed to reduce it expression, although a trend of down-regulation was observed. Resveratrol significantly diminished COX-2 mRNA levels at each tested concentration.

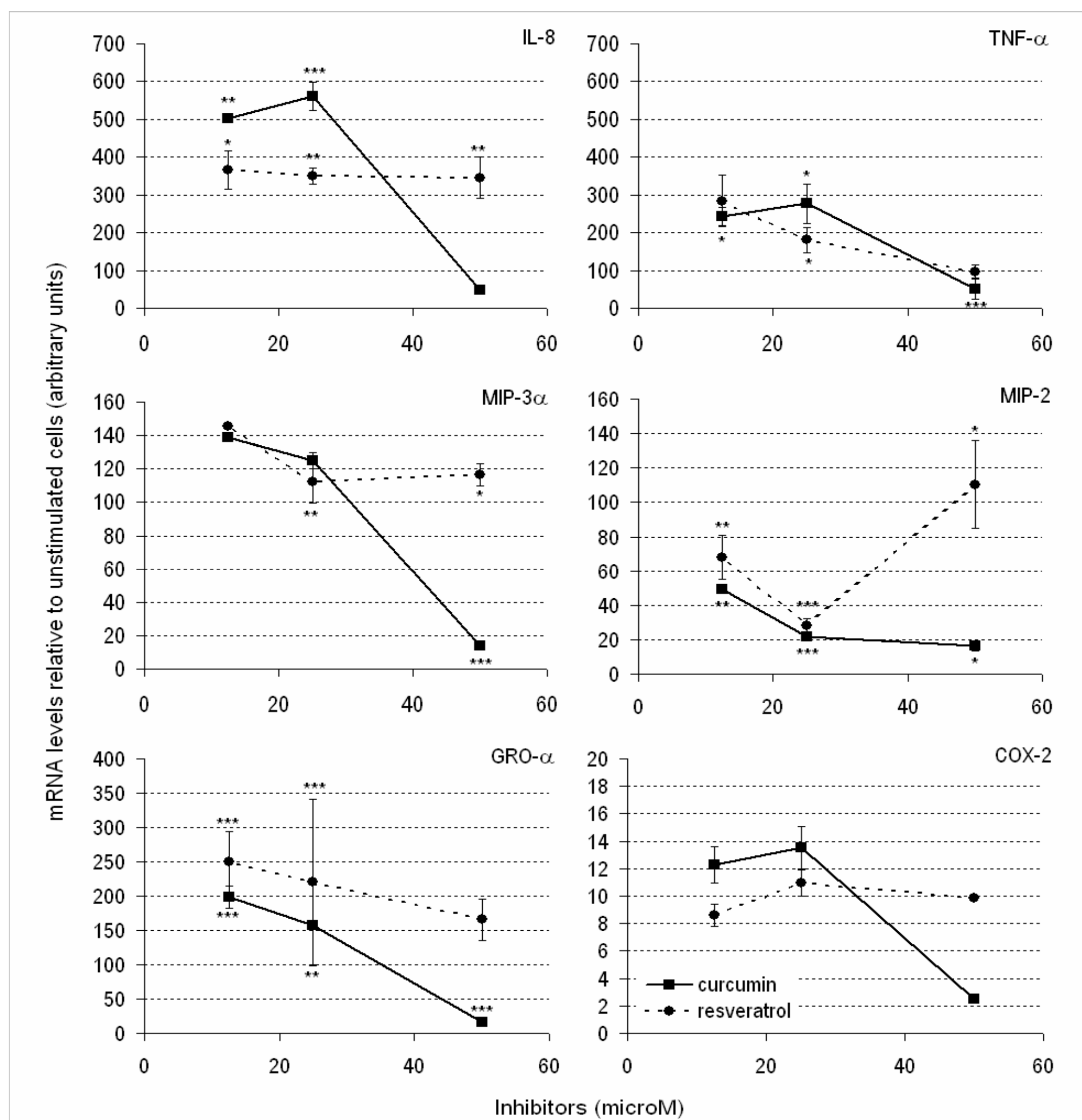


Figure 29: Dose-dependent effects of curcumin and resveratrol on several inflammatory genes in HT29 cells

TNF-α-stimulated cells were treated with various concentrations of curcumin or resveratrol for 6 hours. mRNA levels were determined relative to unstimulated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to stimulated cells.

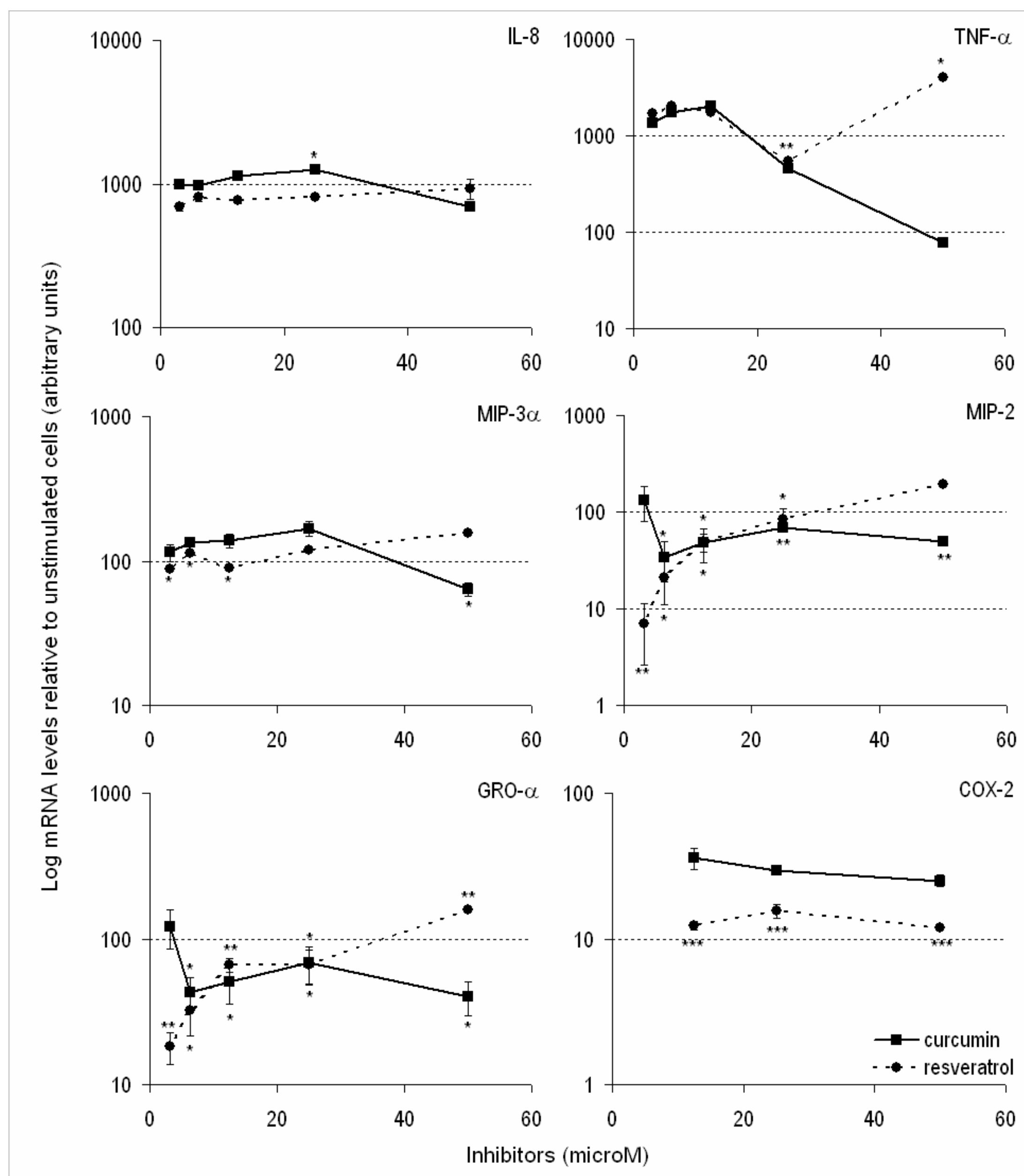


Figure 30: Dose-dependent effects of curcumin and resveratrol on gene expression in T84 cells

Cells were stimulated with TNF-α (100 ng/mL) and LPS (1 μg/mL) in the presence of varying concentrations of curcumin or resveratrol for 6 hours. mRNA levels were quantified relative to unstimulated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to stimulated cells.

DISCUSSION

In this study, we analyzed the anti-inflammatory effect of curcumin and resveratrol on the production of chemokines in intestinal inflammation *in vitro*. Human colon adenocarcinoma cells HT29 and T84 were stimulated mainly with TNF-α and the anti-inflammatory effects of candidate compounds determined on the expression of several genes involved in inflammatory processes and on the production of chemokines (IL-8 and MIP-3α) and PGE_2. Curcumin and resveratrol had no effect on the production of IL-8, however, an effect was observed on the gene expression. Furthermore, the production of MIP-3α was strongly affected by curcumin whereas a weak down-regulation of its expression was obtained with both curcumin and resveratrol. The generation of PGE_2 was also inhibited by curcumin and resveratrol. These observations give insight into the different mode of action of phenolic compounds on colon cancer cell lines.

During intestinal inflammation, a large number of inflammatory cells, including neutrophils and lymphocytes, infiltrate the mucosa, lamina propria, and the crypts. Transmigration of neutrophils across the epithelium leads to a structural and functional compromise in epithelial integrity and alteration in barrier function (Madara, 1997; Nusrat et al., 1997). Therefore, compounds that are able to block neutrophil migration can ameliorate inflammation. Curcumin and resveratrol did not inhibit the production of IL-8 in epithelial cells, however, a weak effect was observed on the gene level where curcumin and resveratrol down-regulated IL-8 mRNA only at high concentration. Epithelial cells also express different chemokines belonging to the same CXC subfamily as IL-8, including GRO-α, GRO-γ and MIP-2 (van Deventer, 1997). Since they also recruit neutrophils, an inhibitory effect on their expression results in a lesser mucosal neutrophil immigration. In addition, IL-8, GRO-α, GRO-γ and MIP-2 share the same receptor CXCR2. Although curcumin and resveratrol did not inhibit the production of IL-8, we suggest that they might affect neutrophil attraction through the down-regulation of GRO-α, GRO-γ and MIP-2.

Furthermore, lymphocyte chemoattraction is associated with increased levels of MIP-3α. Human intestinal epithelial cells regulate the expression of this chemokine which is responsible to recruit APC (e.g. DC) and memory T cells in addition to develop the host adaptive immune response (Izadpanah et al., 2001). An increase in the production of this chemokine was found in primary epithelial cells isolated from patient with IBD, suggesting that “inflamed” epithelial cells produce and secret MIP-3α which attracts CD4+ T lymphocytes and immature DCs to the inflamed epithelium (Kwon et al., 2002). Compounds acting on this level are able to reduce the recruitment of lymphocytes, and thus, inflammatory processes involved

in IBD. Curcumin and resveratrol differ in their mode of action on the production of MIP-3α. While resveratrol had no effect on (T84), or rather enhanced (HT29), the MIP-3α production in epithelial cells, curcumin inhibited it in both cell lines tested. With regard to curcumin, our results suggest that inhibiting MIP-3α it may affect the lymphocyte infiltration, meliorating inflammatory symptoms.

Moreover, the potency of curcumin and resveratrol to reduce the generation of PGE_2 was analyzed. As described before, PGE_2 is expressed by epithelial cells in response to a pathogen or TNF-α stimulation (Singer et al., 1998). In IBD where the level of this cytokine is increased (D'Haens, 2003), TNF-α induces the generation of COX-2-dependent PGE_2 which, in turn, is responsible for the epithelial chloride secretion, and thereby, IBD-associated diarrhea (Singer et al., 1998). In our study, curcumin and resveratrol down-regulated COX-2 expression (at 50 μM) in HT29 and T84 cells, and PGE_2 secretion in HT29 cells. By reducing COX-2-dependent PGE_2 production, curcumin and resveratrol may ameliorate diarrhea present in CD. Moreover, we speculate about the possibility that curcumin and resveratrol could inhibit chloride secretion through the inhibition of TNF-α.

In conclusion, we showed that natural substances are able to attenuate inflammation in intestinal epithelial cells by modulating the expression of pro-inflammatory genes and MIP-3α production. Based on the obtained results, curcumin was more potent than resveratrol with a larger panel of effects. The effects of resveratrol on intestinal epithelial cells were not consistent in this study. Discrepancies between chemokine production and gene expression were observed. Furhter analyses should be done in order to clarify its effect on epithelial cells.

GENERAL DISCUSSION

The intestinal epihtelium is normally exposed to a large number of microorganisms (e.g., bacteria, viral agents) and diverse types of nutrients. The latter are able to cross the interface between the gut lumen and the intestinal mucosa, while the crossing of potentially injurious agents is effectively and efficiently prevented. Such an event is only possible due to the presence of highly sensitive intestinal immune system which regulates the intestinal host defense and supports epithelial cells in their primordial function as maintainers of the epithelial integrity. Once this regulatory balance between pathogens, epithelial cells and immune system is disturbed, nonspecific stimulation and activation lead to the release of increased amounts of potent destructive molecules, leading to tissue injury (MacDermott, 1994).

One of the worldwide most prevalent intestinal disorders is IBD, a chronic disease arising from a dysregulated immune response. Several immune cells including macrophages, T and B cells are present in high amounts in the inflamed mucosa producing inflammatory mediators such as cytokines, chemokines, prostaglandins in addition to immunoglobulins (e.g., IgA) (MacDermott, 1994). Although different cytokines are present in IBD, TNF-α still the most relevant. It is found through the entire course of the disease and induces the secretion of a variety of proinflammatory cytokines and chemokines from epithelial and mononuclear cells (Sands, 2004), thus enhancing the inflammatory response. Since several factors could be responsible for the onset of IBD (e.g., genetic, environmental, bacteria invasion) (Fiocchi, 1998), actual therapeutic drugs, including corticosteroids, aminosalicylates, immunomodulators and monoclonal antibodies (anti-TNF) are used only to attenuate symptoms and/or relapses of the disease. In addition, the use of such medicaments lead to marked site effects (Yang and Lichtenstein, 2002).

Natural substances with anti-inflammatory properties could assist aforementioned therapies by reducing inflammatory processes and delaying the relapse of the disease. In addition, they could be useful in the prevention of IBD. In this thesis, the anti-inflammatory effects of three natural compounds (EGCG, curcumin and resveratrol) and two plant extracts (BS and RO) were analysed in an *in vitro* model of intestinal inflammation (chemical structure of the substances are shown in Figure 31). Human colon adenocarcinoma cell lines, HT29 and T84, were used as a human *in vitro* cell culture model with close resemblances to the human intestinal epithelium. Although several groups, including ours, have used these cells as *in vitro* model for intestinal inflammation, it should be emphasized that both cell lines have a neoplastic origin which differ from human intestinal epithelium features, e.g., neoplastic cells growth faster than normal cells and are more sensitive to toxins.

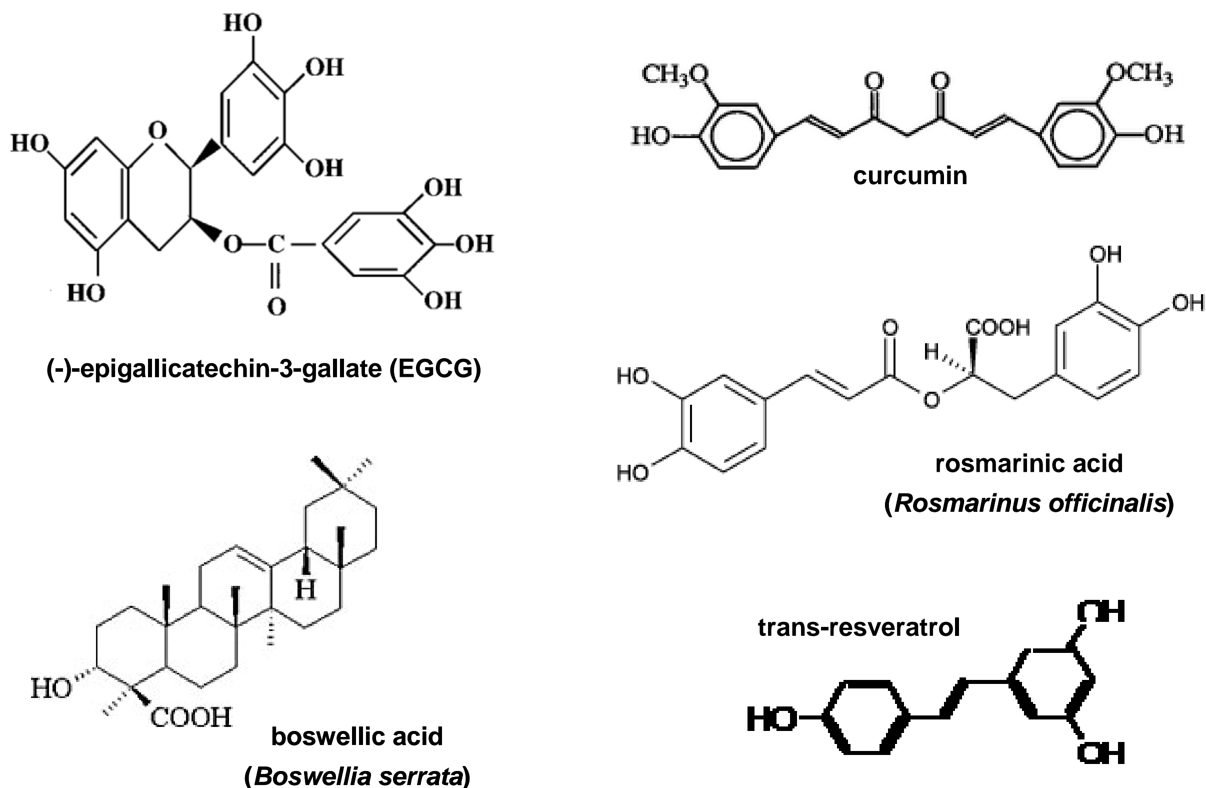


Figure 31: **Chemical structure of tested substances**
EGCG: http://www.biochemj.org/bj/368/0695/bj3680695.htm;
Boswellic acid: http://www.itmonline.org/arts/myrrh.htm;
Curcumin: http://parasitology.informatik.uni-wuerzburg.de;
Rosmarinic acid: http://www.alexis-corp.com;
Trans-resveratrol: http://www.wine.yamanashi.ac.jp/biofunctional/biofunctional.html.

The effects of candidate substances were determined at various levels: (1) on the expression of genes induced by TNF-α; (2) on the secretion of metabolites that are associated with inflammation; and (3) on the protein expression. Table 5 summarizes the anti-inflammatory effect of tested compounds in stimulated HT29 and T84 cells.

Table 5: Summary of the effects of candidate substances in intestinal inflammation *in vitro*.

Process	Acronym	HT29					T84		
		EGCG[a]	BS[b]	RO[b]	Curc[a]	Res[a]	EGCG[a]	Curc[a]	Res[a]
Viability		Ø	†	Ø	†	Ø	Ø	Ø	Ø
Gene expression	IL-8	+++	++++	++++	+++	+	+	++	+
	TNF-α	+++	+++	+++	+++	++	++	++++	+
	MIP-3α	+++	++++	+++	++++	+	+++	+++	++
	MIP-2	+++	++++	+++	+++	++	++	+++	++
	GRO-α	+++	++++	++	+++	●	+	+++	++
	GRO-γ	++	+++	+++	+++	±	++	+++	±
	IP-10	++	++	++++	ND	ND	±	ND	ND
	RANTES	++	+	+++	ND	ND	ND	ND	ND
	COX-2	++	++	+++	+++	++	++	++	+
	5-LOX	±	±	±	ND	ND	ND	ND	ND
Metabolite	PGE_2	♣♣♣	ND	ND	♣♣	♣♣♣	-	-	-
	IL-8	♣♣♣ ►	♣♣♣ ►	♣♣♣ ►	♣	♣	♣♣♣ ►		
	MIP-3α	♣♣♣♣ ▼	♣♣♣♣ ▼	♣♣♣♣ ▼	♣♣ ▲	▼	♣♣♣♣	♣♣♣ ►	♣♣ ▲
Protein	IκBα	ND	28%[c]; 27%[d]	57%[c]; 42%[d]	ND	ND	ND	ND	ND
	NF-κB p65	ND	93%[c]; 116%[d]	88%[c]; 109%[d]	ND	ND	ND	ND	ND

Legend:

ND	not determined		
IC_{50}	inhibitory concentration of 50%		
Curc	curcumin		
Res	resveratrol		
a	50 μM		
b	50 μg/mL		
c	25 μg/mL	10 min	
d	25 μg/mL	20 min	
Ø	no cytotoxic	LDH activity	< 10%
†	moderate cytotoxic	LDH activity	10 - 20%
++++	total inhibition	gene expression	> 90%
+++	strong inhibition	gene expression	61 - 90%
++	moderate inhibition	gene expression	31 - 60%
+	weak inhibition	gene expression	5 - 30%
±	no effect	gene expression	< 5%
●	gene induction		~100%
♣♣♣♣	total inhibition	metabolite	> 90%
♣♣♣	strong inhibition	metabolite	61 - 90%
♣♣	moderate inhibition	metabolite	31 - 60%
♣	weak inhibition	metabolite	5 - 30%
-	no production	metabolite	< 5%
▼	IC_{50} < 6 μM	metabolite	
►	IC_{50} between 10 - 20 μM	metabolite	
▲	IC_{50} > 50 μM	metabolite	
	weak increase	metabolite	20 - 25%
	strong increase	metabolite	> 90%
	strong IκBα degradation		
	moderate to weak IκBα degradation		
	amount of NF-κB in the nucleus		

In the course of intestinal inflammation, several mediators including cytokines and chemokines are expressed and released by different cell types including immunocytes and epithelial cells. Cytokines (e.g., TNF-α and IL-1β) are responsible to activate and induce the synthesis of chemokines in epithelial cells (Dionne et al., 1998; Sands, 2004) which then chemoattract leukocytes and lymphocytes to the injured tissue (Ajuebor and Swain, 2002). Data presented in this thesis demonstrate that the synthesis of two important chemokines, IL-8

and MIP-3α, is strongly inhibited by EGCG (IC_{50} of 10.7 and 2.0 μM, respectively), BS (IC_{50} of 11.6 and 2.4 μg/mL, respectively) and RO (with IC_{50} of 20.0 and 3.6 μg/mL, respectively) in **HT29 cells**. Curcumin and resveratrol moderately to weakly affected chemokine production. Interestingly, resveratrol drastically increased the secretion of MIP-3α in these cells, an observation which can not be explained by altered cell viability. In **T84 cells**, EGCG had a similar effect on the chemokine production. Furthermore, curcumin strongly inhibited MIP-3α production with an IC_{50} of 10.5 μM while a weak effect was observed on the IL-8 synthesis. With regard to resveratrol, a moderate to weak effect was observed on the chemokine production. Although EGCG, BS and RO exhibit similar effects on the chemokine secretion, their chemical structure differ from each other. However, their bioavailability could be similar. A similarity in the structure was observed between rosmarinic acid, the active substance of RO, and curcumin, however, the former presents a caffeoyl ester group which may be responsible for its stronger inhibitory effect. A common feature between these substances is their anti-oxidant properties which might explain most of the effects, a statement based on a large number of literature data. Moreover, these results were corroborated by gene expression profiling where EGCG, BS, RO, and curcumin showed potent effects on the expression of genes of the inflammatory pathway, especially those of the cytokine/chemokine network (IL-8, TNF-α, MIP-3α, MIP-2, GRO-α, GRO-γ, and IP-10). In **HT29 cells**, EGCG, BS, RO, and curcumin at high concentrations showed similar effects on the expression of inflammatory gene. With regard to resveratrol, it had a weak or no effect on related genes. In **T84 cells**, curcumin had the strongest effect while EGCG and resveratrol moderately affected the abovementioned genes. We infer that the differences observed between HT29 and T84 reflect different origin of the cells (see Chapter 1).

As described in Chapters 1 and 3, EGCG, BS, and RO strongly affected chemokines belonging to the CXC family and responsible to recruit leukocytes (mainly neutrophils) to the injured tissue, i.e. IL-8, GRO-α, GRO-γ, and MIP-2. These target the receptor CXCR2 (Murphy et al., 2000). Furthermore, EGCG, BS, and RO also inhibited MIP-3α, a member of the CC subfamily involved in the chemoattraction of lymphocytes, DC and monocytes to the “inflamed” tissue. It is a ligand to the chemokine receptor CCR6 (Murphy et al., 2000; Izadpanah et al., 2001). Data presented in this thesis suggest that EGCG, BS, and RO impair the recruitment of immunocytes to the injuried area by affecting biological activities mediated through CXCR2 and CCR6. Analyses of the expression of these receptors on epithelial cells are needed to clarify these hypotheses.

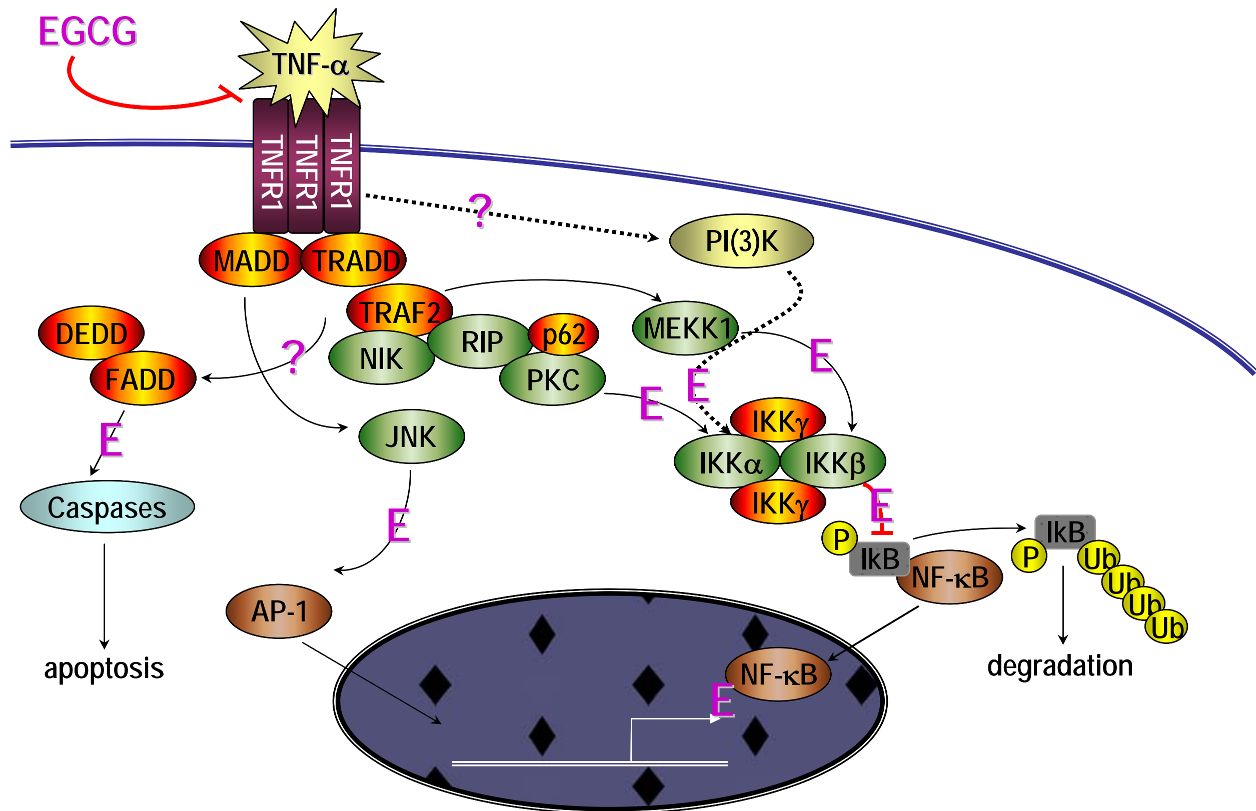
Recently, Wallace and Devchand reviewed the role of COX-2 in gastro-intestinal mucosal defense. COX-2 was rapidly and substantially up-regulated after induction of colitis in

a rat model which was accompanied by a sharp increase of PGD_2, an anti-inflammatory prostaglandin that dampened granulocyte infiltration. In agreement with this, it was reported that treating experimental colitis with selective COX-2 inhibitors during the early phase leads to enhanced granulocyte infiltration and further ulcer development (Wallace and Devchand, 2005). We observed that the expression of COX-2 was elevated in the presence of the stimulus. However, at high concentrations all substances tested inhibited its expression. Moreover, since COX-2 is a key enzyme involved in the generation of PGE_2, the effect of candidate compounds on PGE_2 production was analysed. A moderate to strong inhibition was observed in TNF-α-activated HT29 cells in the presence of EGCG, curcumin and resveratrol, confirming the abovementioned effects on the COX-2 expression. BS and RO were not tested in this assay and T84 cells did not produce detectable amounts of PGE_2. As previously described, EGCG, curcumin and resveratrol inhibited COX-2 which explained its effect on both COX-2 and PGE_2. As reviewed by Wallace, COX-2 is necessary for the GI mucosal defense, however, in a limited amount. Candidate substances reversed TNF-α-induced COX-2 expression and further PGE_2 generation in epithelial cells, suggesting that these could be an undesired property of the substances.

The activation of NF-κB is an important step for the transcription of inflammatory genes, including chemokines. As described in Chapter 3, in unstimulated cells, NF-κB is retained in the cytoplasm bound to IκB. Upon activation, IκB is phosphorylated and degraded allowing NF-κB dissociation and translocation into the nucleus. We showed that BS did not prevent IκBα degradation and did not impair the translocation of NF-κB into the nucleus. On the other hand, RO reduced the IκBα degradation (57% and 42% after 10 and 20 min, respectively); however, no effect was observed on the NF-κB translocation. Since RO affected NF-κB DNA binding (as shown in our EMSA results), we infer that RO may be able to covalently alter NF-κB binding sites, indicating that one mechanism of action of RO is through the NF-κB pathway.

Moreover, we presented in Chapter 2 that EGCG also modulates the expression of multiple genes involved in specific pathways, including those of the immune response, apoptosis, cell adhesion, ECM, growth signaling and cell cycle. Genes of the immune response which were up-regulated with TNF-α and involved in the perpetuation of inflammation were inhibited by EGCG, such as cytokines and chemokines. EGCG also regulated genes of the NF-κB pathway such as IκBα which would not be degradated by the proteasome, maintaining NF-κB in the cytoplasm. Genes involved in apoptosis were either up- or down-regulated in the presence of EGCG. The effects of the latter were heterogeneous, in one hand inhibiting genes of the TNF/TNFR1 signaling and, in the other hand, affecting death

receptors and further the caspase cascade. We suggest that in order to balance the immune system, EGCG may act in cell surveillance, increasing Bcl3 and MCL1; and apoptosis by up-regulating death receptors such as DR5 and DR6. Furthermore, genes involved in cell adhesion were also regulated by EGCG. Our results infer that EGCG is able to affect immunocyte infiltration, and thus, ameliorate inflammation through two ways: inhibiting the production of chemokines, and reducing the expression of adhesion molecules such as ICAM-1, ICAM-4. In addition, EGCG may increase mucosal barrier functions by augmenting the quantity and/or activity of gap- and tight-junctions between adjacent epithelial cells. Moreover, genes responsible for tissue repair and remodeling were elevated by EGCG in activated cells, including LAM5 and collagen. In summary, these data clearly indicate that EGCG, with its heterogeneous effect on several cellular processes, influences specific sets of TNF-α-inducible genes. Beside the anti-inflammatory effects, EGCG may be useful in the regulation of lipid metabolism as well as in cancer therapies. A possible model for the mechanism of action of EGCG, based on DNA microarray data, is out-lined in Fig. 32. Although an array of effects were seen in the gene level in cells treated with EGCG, these data should be further analysed by RT-PCR and protein expression in order to confirm our statement.



Legend:

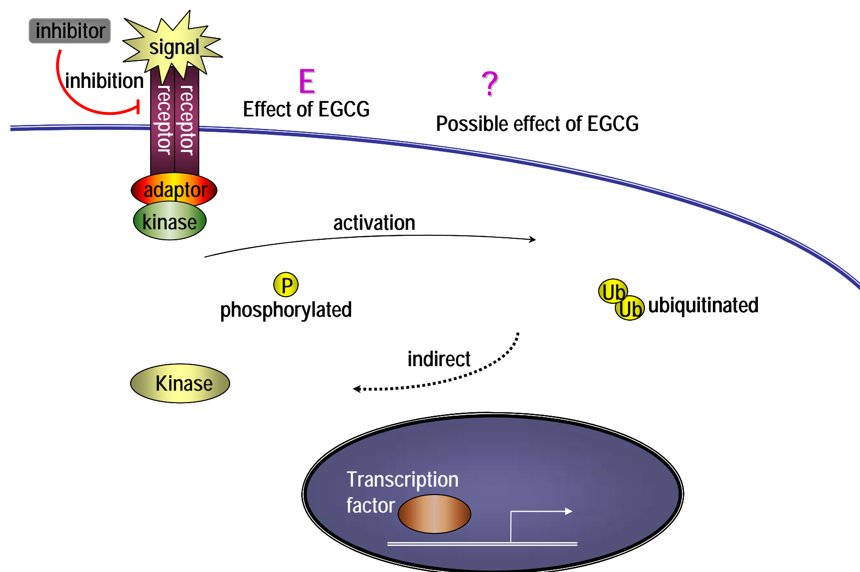


Figure 32: Possible sites of action of EGCG in HT29 cells EGCG interferes with the binding of TNF-α to TNFR1. Moreover, EGCG possibly modulates genes between TRADD and FADD. Apoptosis is impaired by EGCG through the regulation of the caspase cascade. EGCG affects Jun kinase (JNK) and PKC pathways. EGCG may also interfere with the PI(3)K kinase pathway, which would not lead to the activation of IκB kinases (IKK). EGCG induces the expression of MEKK1, which activates IKK, leading to IκB degradation and further NF-κB activation. EGCG directly inhibits IκBα expression. Finally, EGCG may be able to covalentely bind to the NF-κB in the nucleus. NF-κB can not bind to its promoter region which would not activate the expression of inflammatory genes.

Taken together, the findings presented in this thesis provide an extension of the mode of action of phenolic substances in intestinal inflammation *in vitro*. EGCG, BS, RO, and curcumin exert their anti-inflammatory effects by inhibiting the expression and production of chemokines and reducing the generation of PGE_2 (Fig. 33). Furthermore, some specific pathways are affected by EGCG and RO, including TNF/TNFR1 and NF-κB, respectively. More experiments are needed to rule out the effect of EGCG on NF-κB pathway in the protein level. Based on these results, phenolic compounds have the ability to interfere with *in vitro* intestinal inflammation. Obviously, *in vivo* studies are mandatory to corroborate this statement. Provided these prove to be conclusive, phenolic substances will be further applicable for the prevention and attenuation of intestinal inflammatory disorders.

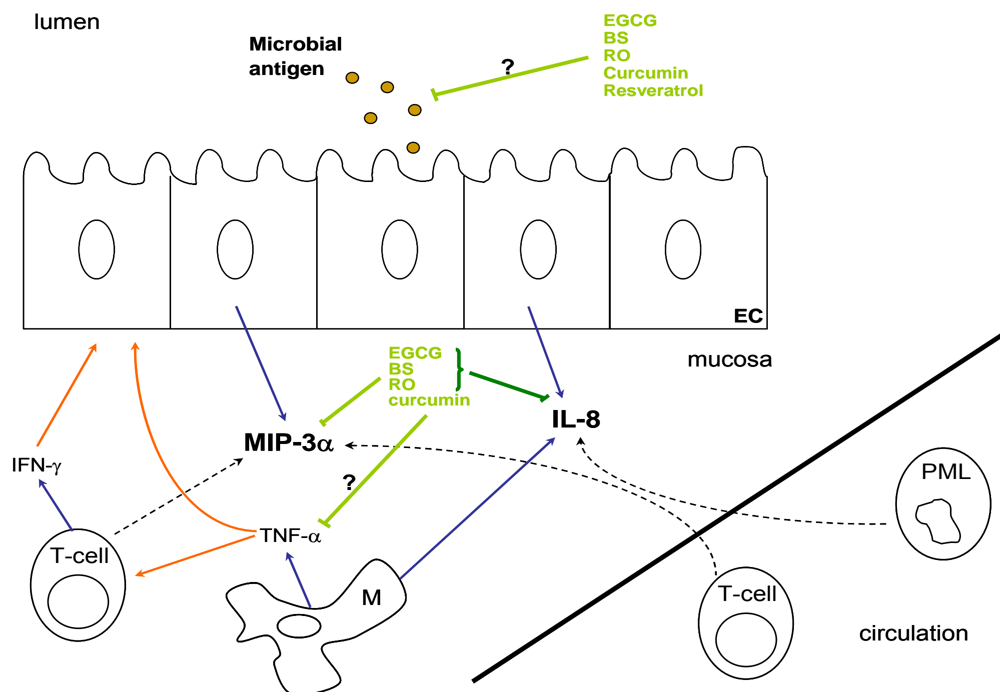


Figure 33: Model for the mode of action of phenolic compounds and plant extracts in the "inflamed" intestinal environment

Dysregulation in the immune response leads to epithelia injury which facilitates the penetration of microbial antigens into the mucosa. Intestinal macrophages initiate the synthesis of TNF-α and IL-8. The former stimulates T-cells to produce IFN-γ, and together, they diminish mucosal barrier functions. TNF-α also activates epithelial cells to produce IL-8 and MIP-3α. High amounts of IL-8 present in the mucosa leads to the recruitment of PML while increased levels of MIP-3α recruit basically lymphocytes from the circulation. EGCG, BS, RO, and curcumin inhibit MIP-3α (as shown in light green) while only EGCG, BS, and RO reduce the production of IL-8 (shown in dark green). It still unclear whether phenolic compounds could also impair the production of TNF-α, although evidences from gene expression data would suggest that they do. It also remains unknown if these substances could directly act on microbial antigens.

OUTLOOK

Gene expression profiling has permitted us to extend our view of the possible effects of EGCG. Although a speculation about the mechanism of action of EGCG has been ilustrated, to complete the knowledge of signaling pathways and to corroborate findings presented in this thesis, genes found to be highly or even not regulated with the stimulus and the treatment should be further analyzed by RT-PCR and also protein expression. Moreover, a time-dependent gene chip experiment could provide data on early, intermediate, and late effects of the substances, delivering more information about the kinetics of specific genes and how they are influenced by the compounds.

The effect of EGCG on genes involved in the NF-κB pathway was quite promising. However, we could not conclude whether these effects would also be observed on the protein level. Studies regarding protein degradation and phosphorylation would give new insights for the action of EGCG.

Overall, there are relatively few *in vivo* data about the anti-inflammatory effects of phenolic substances in intestinal inflammation. In order to use these substances in the clinic, *in vivo* or *ex-vivo* studies are necessary. Furthermore, efficacy studies and “molecular safety” approaches regarding the effects of such substances in other tissues (e.g. liver) should also be addressed.

Since our findings presented in Chapter 3 refered to the effect of whole plant extracts, the isolation and further purification of their active compounds should be taken in consideration. Further investigations on their efficacy and anti-inflammatory activity will give a new insight in the intestinal inflammation field.

As described in Chapter 4, discrepancies to published data were observed regarding the effects of curcumin and resveratrol on the chemokine production and gene expression profile. The reason for these differences is unclear, but they could be due to different experimental conditions (e.g. source of cell line, origin of substances, condition of stimulation). These need to be established in additional experiments.

Additionally, preliminary studies on transporter genes identified EGCG and curcumin as modulators of ABCB1 and ABCC2. The presence of EGCG or curcumin seem to affect the efficacy in importing or exporting xenobiotics to the lumen as well as to prevent the import or export of compounds such as chemotherapeutics into the cell. Additional studies are needed in order to clarify these findings also in the protein level.

It may be difficult for humans to consume sufficient quantities of phenolic compounds from their normal diets. This gives the reason for enhanced supplementation by fortifying foods or using supplements. Animal studies and human trials would indicate a reasonable safety profile that will help to define and calculate the ideal dose for a chemically synthesized or highly purified “natural drug” of known quality.

SUMMARY

Natural plant extracts and their isolated phenolic substances have been shown to exhibit several functions including the anti-inflammatory activity in various tissues. EGCG, curcumin and resveratrol have been identified as possible anti-inflammatory agents while little is known about extracts of *Boswellia serrata* (BS) and *Rosmarinus officinalis* (RO).

In this thesis project, several methods were applied in order to identify the anti-inflammatory effects of aforementioned substances and extracts in “inflamed” intestinal epithelial cells. The colon cancer cell lines, HT29 and T84, were stimulated with TNF-α in the presence of various concentrations of EGCG, BS, RO, curcumin and resveratrol. Their effects were analyzed at different levels: (1) on the expression of multiple genes involved in inflammatory responses; (2) on the generation of metabolites such as PGE_2, and production of chemokines (e.g., IL-8 and MIP-3α); (3) and on the protein expression. The expression of genes was determined by quantitative RT-PCR and microarray technology. The production of IL-8, MIP-3α and PGE_2 were measured by ELISA. The expression of proteins belonging to the NF-κB pathway was analyzed by immunoblots. In addition, NF-κB DNA binding was carried out using EMSA. Treatment of activated cells with EGCG, BS, RO, curcumin and resveratrol reduced the expression of most genes related to the inflammatory response of HT29 and T84 cells. Furthermore, EGCG, curcumin and resveratrol diminished the generation of PGE_2 in HT29 cells. In addition, the secretion of IL-8 and MIP-3α were dose-dependently inhibited by candidate compounds in HT29 cells. EGCG reduced the production of these chemokines also in T84 cells while curcumin and resveratrol only diminished MIP-3α synthesis. Moreover, the expression of IκBα and NF-κB p65 were analyzed in HT29 cells. BS was not able to prevent IκBα degradation and subsequent NF-κB translocation whereas RO interfered with IκBα degradation, however, it did not impede the activation of NF-κB. Additionally, RO reduced NF-κB DNA binding tested by EMSA.

Collectively, the presented data show that phenolic substances or whole plant extracts have the capacity to attenuate inflammatory features by modulating genes involved in inflammatory responses; by reducing the chemoattraction of immunocytes through the inhibition of IL-8 and MIP-3α; and by affecting specific pathways involved in inflammation such as the NF-κB. Furthermore, phenolic compounds might have a preventive and/or therapeutic utility in chronic inflammation such as IBD, and therefore, they should be further analyzed in *in vivo* studies to consolidate these findings.

ZUSAMMENFASSUNG

Natürliche Pflanzen-Extrakten und deren phenolische Substanzen haben verschiedene Funktionen einschliesslich entzündungshemmende Effekte in verschiedene Gewebe. Während EGCG, Curcumin und Resveratrol als mögliche anti-inflammatorische Substanzen nachgewiesen wurden, ist über *Boswellia serrata* (BS) und *Rosmarinus officinalis* (RO) Extrakten wenig bekannt.

In diese Dissertation wurde verschiedene Methoden angewendet, um die entzündungshemmenden Effekte der erwähnten Substanzen und Extrakten in Darmepithelzellen zu identifizieren. Die Darmkrebs-Zelllinien HT29 und T84 wurden mit TNF-α zusammen mit verschiedenen Konzentrationen von EGCG, BS, RO, Curcumin und Resveratrol stimuliert. Ihre Effekte wurden auf unterschiedlichen Ebenen analysiert: (1) Expression der mehrfachen Gene die in entzündliche Antworten beteiligt werden; (2) Bildung der Stoffwechselprodukte wie PGE_2, und die Freisetzung von Chemokinen (z.B. IL-8 und MIP-3α); (3) und Proteinexpression. Die Expression der Gene wurde durch quantitative RT-PCR und DNA microarray Technologie evaluiert. Die Produktion von IL-8, MIP-3α und PGE_2 wurde durch ELISA gemessen. Die Expression der Proteine, die zum NF-κB Pathway gehören, wurde durch Immunoblots analysiert. Zusätzlich wurde auch die NF-κB DNA Bindung durch EMSA geprüft. Aktivierte Zellen, die mit EGCG, BS, RO, Curcumin und Resveratrol behandelt wurden, haben die Expression der meisten analysierten Entzündungs-Gene in HT29 und T84 reduziert. Ausserdem haben EGCG, Curcumin und Resveratrol die Bildung von PGE_2 in HT29 Zellen vermindert. Zusätzlich wurde die Freisetzung von IL-8 und MIP-3α dosis-abhänging durch die getesteten Substanzen gehemmt. EGCG verringert die Produktion dieser Chemokine auch in den T84 Zellen, während Curcumin und Resveratrol nur die MIP-3α Freisetzung vermindern. Weiterhin wurde auch die IκB-α Expression in HT29 analysiert. BS konnte den IκB Abbau und nachfolgende NF-κB Translokation nicht verhindern, während RO störend auf den IκB Abbau wirkte. RO konnte die NF-κB DNA Bindung reduzieren.

Die in dieser Arbeit dargestellten Daten zeigen, dass phenolische Substanzen oder Gesamt-Extrakten anti-inflammatorische Eigenschaften aufweisen, indem sie die Gene, die in entzündliche Antworten betroffen sind, modulieren; die Chemoattraktion von Immunocyten durch die IL-8 und MIP-3α Inhibition reduzieren; und spezifischen Pathways beeinflussen, die in Entzündung mit eingezogen sind wie NF-κB. Somit können phenolische Substanzen eine vorbeugende und/oder therapeutische Wirkung in chronischen Entzündungen wie IBD haben. Diese Befunde soltten nun durch *in vivo* Studien bestätigt werden.

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02/1991 - 11/1997	**High School**, Blumenau, Brazil Abitur
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03/2001 - 06/2001	Pharmacy Sesi, Florianópolis, Brazil
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Technical Skills:

Isolation and identification of natural compounds

HPLC, paper chromatography, thin-layer chromatography (TLC), column chromatography (CC), Isolation and spectroscopic analysis of natural substances

Molecular biology and gene expression

Western blotting and electrophoresis, RT-PCR (Taqman^{TM}), Microarray technology, Isolation of RNA

Biochemical analysis

ELISA, Nitric Oxide-determination (Griess reaction), FACScan Analyse

Cell culture and cell biology

Experimental work with different cell lines (THP-1, RAW 264.7, HT29, T84, Caco-2)
Isolation of human polymorphonuclear leukocytes (PMNL)
Neutrophil adhesion assay in HT29 cells
Transfection in eukaryotic cells
Immunofluorescence in gastro-intestinal epithelial cells

Animal work

Isolation of mouse intestinal epithelial lymphocytes and peritoneal macrophages
Injections in rats: gavage, IP & SC

Applications in humans

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Portuguese (first language)
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Publications

Porath D, *Riegger C, Drewe J, Schwager J.*
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