Diana Jurado Santos Serra

MODULATION OF INTESTINAL INFLAMMATION BY DIETARY POLYPHENOLS IN COMPARISON WITH 5-AMINOSALICYLIC ACID: AN *IN VITRO* APPROACH

Tese de Doutoramento em Ciências Farmacêuticas, ramo de Bioquímica, sob orientação científica da Professora Doutora Teresa Dinis e da Professora Doutora Leonor Almeida e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro de 2015

· U C ·

UNIVERSIDADE DE COIMBRA

FFUC FACULDADE DE FARMÁCIA
UNIVERSIDADE DE COIMBRA

Diana Jurado Santos Serra

Modulation of Intestinal Inflammation by Dietary Polyphenols in Comparison with 5-Aminosalicylic Acid: an *In Vitro* Approach

Regulação da Inflamação Intestinal por Polifenóis da Dieta em Comparação com o Ácido 5-Aminosalicílico: uma Abordagem *In Vitro*

Tese de Doutoramento em Ciências Farmacêuticas, ramo de Bioquímica, apresentada à Faculdade de Farmácia da Universidade de Coimbra para obtenção do grau de Doutor

Orientadores: Professora Doutora Teresa Dinis e Professora Doutora Leonor Almeida

Coimbra, 2015

Front Cover by João Pinto

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra, para prestação de provas de Doutoramento em Ciências Farmacêuticas, no ramo de Bioquímica.

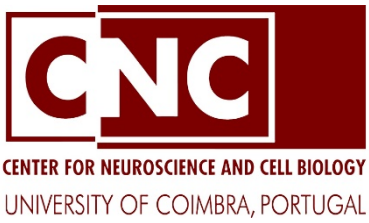
Dissertation presented to the Faculty of Pharmacy, University of Coimbra, in fulfilment of the requirements for obtaining a Doctoral degree in Pharmaceutical Sciences, branch of Biochemistry.

Este trabalho foi realizado na Faculdade de Farmácia, no âmbito das atividades do Centro de Neurociências e Biologia Celular de Coimbra, Universidade de Coimbra, Coimbra, Portugal, sob orientação científica da Professora Doutora Teresa Dinis e da Professora Doutora Leonor Almeida.

A realização deste trabalho foi apoiada pela Fundação para a Ciência e Tecnologia (bolsa de Doutoramento: SFRH/BD/7541/2010 e projectos: PTDC/SAU-OSM/102907/2008 e PEst-C/SAU/LA0001/2013-2014), pelo Fundo Europeu de Desenvolvimento (FEDER) através do COMPETE - Programa Operacional Factores de Competitividade (POFC) e pelo Projecto Estratégico 2015: UID / NEU / 04539 / 2013.

This work was performed at the Faculty of Pharmacy, within the activities of the Center for Neuroscience and Cell Biology of Coimbra, University of Coimbra, Coimbra, Portugal, under the supervision of Professor Teresa Dinis and Professor Leonor Almeida.

The execution of this work was supported by the Portuguese Foundation for Science and Technology (Ph.D. grant: SFRH/BD/7541/2010 and projects: PTDC/SAU-OSM/102907/2008 and PEst-C/SAU/LA0001/2013-2014), by FEDER funds through the Operational Programme Competitiveness Factors - COMPETE and by the Strategic Project 2015: UID / NEU / 04539 / 2013.



UNIVERSIDADE DE COIMBRA

Aos meus pais,

ao João,

ao Sebastião

Agradecimentos

O trabalho apresentado nesta dissertação não teria sido possível sem o inestimável auxílio e encorajamento de algumas pessoas às quais gostaria de expressar vivamente a minha gratidão:

À Doutora Leonor Almeida, minha orientadora, pela sua amizade, generosidade e constante incentivo, e ainda pelo apoio científico, ensinamentos e orientação prestados, os quais foram determinantes para o meu crescimento pessoal e profissional ao longo destes anos.

À Doutora Teresa Dinis, minha orientadora, pelo seu interesse e permanente disponibilidade com que sempre acedeu prestar esclarecimentos e auxílio na resolução de numerosos problemas práticos durante esta jornada e também pelas sugestões que contribuíram para o enriquecimento do trabalho experimental e da elaboração do texto.

À Doutora Alexandrina Mendes e sua doutoranda Ana Rufino agradeço a ajuda prestada nos ensaios de PCR, tanto na otimização das condições experimentais como na análise dos resultados obtidos.

Queria ainda agradecer à Doutora Margarida Caldeira e Doutora Luísa Cortes pela ajuda preciosa na utilização do microscópio confocal.

Às minhas colegas de laboratório Mariana e Sónia pelo seu companheirismo e boa disposição que foram fundamentais para levar este trabalho a bom porto e, especialmente à Joana e à Carla, queria agradecer a grande ajuda que me deram nos primeiros tempos de laboratório e por todas as dicas e conselhos que muito me incentivaram ao longo deste árduo caminho.

Gostaria ainda de agradecer aos meus pais pelo apoio incondicional, pela paciência e por todos os sábios conselhos que me têm dado ao longo destes últimos anos assim como durante toda a vida.

Por último, ao meu marido pela paciência e ajuda na aprimoração das imagens para publicação, pela enorme contribuição na formatação deste trabalho bem como pela excelente selecção e edição das imagens constantes na capa da presente tese e acima de tudo pela enorme generosidade e amor que todos os dias me dá.

Table of Contents

Abbreviations

5-ASA	5-aminosalicylic acid
AMPs	Antimicrobial peptides
ANOVA	One-way analysis of variance
AP-1	Activator protein-1
APCs	Antigen-presenting cells
APRIL	Proliferation-inducing ligand
ARE	Antioxidant response elements
BAFF	B-cell activating factor
BSA	Bovine serum albumin
C3G	Cyanidin-3-glucoside
CAT	Catalase
CD	Crohn's disease
cDNA	Complementary DNA
COX-2	Cyclooxygenase-2
DAN	2,3-diaminonaphthalene
DCs	Dendritic cells
DCF	2′,7′-dichlorofluorescein
$DCFH_2$-DA	2′,7′-dichlorodihydrofluorescein diacetate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
DTT	Dithiothreitol
DUSPs	Dual-specificity phosphatases
ECGG	Epigallocatechingallate
EDTA	Ethylenediaminetetraacetic acid
EpRE	Electrophile response elements
ERK 1/2	Extracellular signal-regulated kinases 1 and 2
FAE	Follicle-associated epithelium
FBS	Fetal bovine serum
GALT	Gut-associated lymphoid tissue

GCL	Glutamate cysteine ligase
GCLC	Catalytic subunit of glutamate cysteine ligase
GCLM	Modifier subunit of glutamate cysteine ligase
GPX	Glutathione peroxidase
GSH/GSSG ratio	Ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG)
HEPES	4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid
HO-1	Hemoxygenase-1
HT-29	Human colon cancer cell line
IBD	Inflammatory bowel disease
IECs	Intestinal epithelial cells
IELs	Intraepithelial lymphocytes
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IkB	Inhibitor of kappa B
IKK	IkB kinase
IL	Interleukin
ILCs	Innate lymphoid cells
ILFs	Isolated lymphoid follicles
iNOS	Inducible nitric oxide synthase
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JNKs	c-Jun N-terminal kinases
Keap1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharide
M cells	Microfold cells
MAMPs	Microbe-associated molecular patterns
MAPKs	Mitogen-activated protein kinases
MHC	Major histocompatibility complex
MLNs	Mesenteric lymph nodes
Mn-SOD	Manganese-superoxide dismutase
mRNA	Messenger RNA
MPO	Myeloperoxidase
MTT	3-(4,5-dimethylthiazol-2yl)2,5-diphenyl-tetrazolium bromide
MUC	Mucin

MyD88	Myeloid differentiation primary response 88 protein
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NF-kB	Nuclear factor kappa B
NKT cells	Non-classical natural killer T cells
NLRs	(NOD)-like receptors
NO	Nitric oxide
NOD	Nucleotide oligomerization domain
NOX	NADPH oxidase
NQO1	NAD(P)H:quinone oxidoreductase-1
Nrf2	Nuclear factor erythroid 2
OPT	O-phthaldialdehyde
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIAS	Protein inhibitors of activated STAT
pIgR	Polymeric immunoglobulin receptor
PMSF	Phenylmethylsulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
PPs	Payer’s patches
PRRs	Pattern-recognition receptors
PVDF	Polyvinylidene difluoride
qRT-PCR	quantitative real-time PCR
RA	Retinoic acid
Resv	Resveratrol
RNA	Ribonucleic acid
ROS/RNS	Reactive oxygen and nitrogen species
RXR	Retinoid X receptor
SED	Subepithelial dome
SEMA7A	Semaphorin 7A
SOCs	Suppressors of cytokine signalling
SOD	Superoxide dismutase

STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
TEDs	Trans-epithelial dendrites
TGF-β	Transforming growth factor-β
Th lymphocytes	T-helper lymphocytes
TIR	Toll-interleukin-1 receptor
TJ	Tight junction
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRAM	TRIF-related adapter molecule
Treg Cells	Regulatory T cells
TRIF	TIR domain-containing adaptor protein inducing IFN-β
TRX	Thioredoxin
TSLP	Thymic stromal lymphopoietin
TYR	Tyrosine
UC	Ulcerative colitis

Resumo

Resumo

A Doença Inflamatória Intestinal (DII) é uma patologia de carácter crónico e recidivante que afeta o trato gastrointestinal e cuja etiologia ainda não se encontra completamente esclarecida. Dado que, até ao momento, ainda não foi encontrada a cura para a DII e o recurso ao tratamento farmacológico convencional conduz a resultados que são claramente insatisfatórios, o desenvolvimento de estratégias alternativas que sejam eficazes para prevenir a progressão desta doença continua a constituir objeto de intensa investigação. Por outro lado, os polifenóis da dieta, aliando a sua potencial atividade anti-inflamatória ao seu efeito antioxidante, têm sido considerados promissores na prevenção e tratamento de várias doenças inflamatórias crónicas e, por esta razão, têm recebido particular atenção no caso da DII.

Neste contexto, o trabalho desenvolvido na presente Dissertação teve como objetivo, em primeiro lugar, contribuir para o esclarecimento dos mecanismos moleculares subjacentes à atividade anti-inflamatória de dois polifenóis estruturalmente diferentes, a antocianina cianidina-3-glucósido (C3G) e o estilbeno resveratrol (Resv), normalmente presentes na dieta Mediterrânica, e, em segundo lugar, comparar esses efeitos com os de um fármaco convencional, o ácido 5-aminosalicílico (5-ASA), nas mesmas condições experimentais. Para esse efeito, as células HT-29 foram inicialmente tratadas com os polifenóis, individualmente ou em combinação com o 5-ASA, em concentrações previsivelmente atingíveis no intestino (25 μM no caso dos polifenóis e 500 μM no caso do 5-ASA) e, posteriormente, expostas a uma mistura de citocinas (IL-1α, TNF-α e IFN-γ) durante um determinado período de tempo.

Numa primeira fase deste trabalho, foi demonstrada a capacidade da C3G e do Resv diminuírem quer a produção de mediadores pro-inflamatórios, como o óxido nítrico e a prostaglandina E_2, quer a expressão de enzimas pro-inflamatórias, como a iNOS e a COX-2, sem afetarem, no entanto, a ativação do fator nuclear kB, em células estimuladas pelas citocinas. Contudo, nestas células, a quantidade de STAT1 ativada diminuiu significativamente no núcleo. Foi também demonstrado que o Resv, ao contrário do 5-ASA, inibiu a ativação da JNK, mas nem o Resv nem o 5-ASA foram capazes de inibir a ativação da p38 MAPK nas mesmas células estimuladas.

Numa segunda parte do trabalho, foi investigado o envolvimento das vias do Nrf2 e do PPAR-γ no efeito protetor referido da C3G e do Resv nas células HT-29 sob ação das citocinas, em comparação com o fármaco 5-ASA. Ambos os compostos demonstraram serem capazes de induzir a via do Nrf2, de aumentar a expressão de RNA mensageiro de duas enzimas

importantes, a hemoxigenase-1 e a glutamato cisteína ligase, de aumentar a relação entre o glutatião reduzido (GSH) e o glutatião oxidado (GSSG) e de inibir a produção de espécies reativas nessas células estimuladas pelas citocinas. Adicionalmente, o Resv e o 5-ASA, mas não a C3G, aumentaram a quantidade de PPAR-γ no núcleo das mesmas células estimuladas.

Atendendo à diferença de concentrações usadas neste estudo, os dados obtidos sugerem que tanto a C3G como o Resv possuem uma capacidade anti-inflamatória e antioxidante superior à do fármaco convencional 5-ASA.

Embora os resultados deste estudo *in vitro* não tenham indicado um efeito aditivo ou sinergético entre a associação da C3G ou do Resv com o 5-ASA, a ocorrência desse efeito em modelos animais da doença ou em humanos não pode ser excluída.

Em conclusão, os resultados deste estudo contribuem para o esclarecimento dos mecanismos de ação anti-inflamatória da C3G e do Resv, ao revelarem o seu envolvimento em vias de sinalização celular críticas no processo inflamatório, reforçam o carácter promissor destes compostos como nutracêuticos adjuvantes da terapêutica convencional e abrem caminho à seleção de outros compostos naturais para o combate da doença inflamatória intestinal.

Abstract

Abstract

Inflammatory Bowel Disease (IBD) is a chronic and relapsing disorder of the gastrointestinal tract, whose etiology remains not fully understood. Given that, until now, IBD does not have cure and the results achieved with the conventional pharmacological treatment are clearly unsatisfactory, there is a great interest in exploring alternative strategies effective in limiting IBD progression. On the other hand, dietary polyphenols, allying their putative anti-inflammatory activity to their antioxidant effect, have been considered promising molecules for the prevention and treatment of several chronic inflammatory disorders and, for this reason, they have been receiving particular attention in the case of IBD.

In this context, the work developed in the present Thesis aimed, primarily, to contribute for an extensive clarification of the molecular mechanisms underlying the anti-inflammatory effects of two structurally different polyphenolic compounds, the anthocyanin cyanidin-3-glucoside (C3G), and the stilbene resveratrol (Resv), both present in the Mediterranean diet, and secondly to compare these effects with those of a well-known anti-inflammatory drug, 5-aminosalicylic acid (5-ASA), in the same experimental conditions. With this purpose, a human intestinal cell line (HT-29) was employed and upon cell treatment with each polyphenol *per se* or in combination with 5-aminosalicylic acid (5-ASA), at the concentrations predictably reached at the intestinal level (25 μM for the polyphenols and 500 μM for 5-ASA), the cells were exposed to a combination of cytokines (IL-1α, TNF-α and IFN-γ) for a certain period of time.

In the first phase of this study, it was demonstrated the ability of C3G and Resv to counteract the production of cytokine-induced pro-inflammatory mediators, such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), and the expression of cytokine-induced pro-inflammatory enzymes, namely iNOS and COX-2, without affecting the activation of nuclear factor kB (NF-kB), but decreasing the amount of activated STAT1 in the nucleus of HT-29 cells. It was also shown that, unlike 5-ASA, Resv inhibited the activation of JNK but neither Resv nor 5-ASA were able to inhibit the activation of p38 MAPK in cytokine-stimulated HT-29 cells.

In a second stage of this work, the involvement of Nrf2 and PPAR-γ pathways in the anti-inflammatory action of C3G and Resv were investigated in comparison with 5-ASA. It was demonstrated that both C3G and Resv were able to induce Nrf2 activation, to increase the ARE-regulated mRNA expression of two important enzymes, hemoxygenase-1 (HO-1) and glutamate cysteine ligase (GCL), to enhance the ratio of reduced glutathione (GSH) to oxidized

glutathione (GSSG) and to inhibit the production of reactive species, in cytokine-challenged cells. Furthermore, unlike C3G, Resv and 5-ASA increased the amount of PPAR-γ in the nucleus of these stimulated cells.

Considering the difference in the concentrations used in this study, the obtained results suggest that both C3G and Resv could have a higher anti-inflammatory and antioxidant capacity than the conventional drug 5-ASA.

Although the undertaken *in vitro* studies did not provide evidence to support an additive or synergistic effect resulting from the association of C3G or Resv with 5-ASA, such effect cannot be excluded in animal models of the disease or in human patients.

In conclusion, the results of this study contribute not only to clarify the action mechanisms behind the anti-inflammatory effects of C3G and Resv, by revealing their involvement in key inflammation-related cellular signalling pathways, but also to reinforce the envisagement of these compounds as promising nutraceuticals in combination to conventional drugs and to open windows for the selection of other natural compounds to defeat inflammatory bowel disease.

Chapter 1

General Introduction

General Introduction

1. Intestinal homeostasis - a finely tuned balance

The intestinal compartment constitutes the largest barrier tissue of the human body, with a surface area of approximately 200-400 m^2, which is colonized by about 100 trillion microorganisms, mostly bacteria, collectively called microbiota or commensal flora [1-3]. The quantity and diversity of bacteria vary along the intestine, which is possibly explained by differences in host genetics and by interactions with the external environment [3, 4]. Due to millions of years of co-evolution, the relationship established between the host and the commensal flora is of a symbiotic nature, since the microbiota increases the digestion capacity of the host, helps him in preventing pathogen colonization and contributes to intestinal physiology and immunology, benefiting, in turn, from the nutrients and the mild temperature of the human body [2-5]. However, not neglecting this mutually beneficial relationship, the close proximity with this huge number of microorganisms constitutes a great challenge to the host, who has to tolerate them, avoiding overreactions that, otherwise, could lead to the damage of the intestinal tissue and, at the same time, should remain vigilant against potentially threatening signals derived from the presence of these microorganisms [2-4]. In addition, since the intestine serves as a portal of entry for nutrients into the body, human organism has to cope with a myriad of antigens, frequently ingested through the diet, such as food antigens or environmental toxins or even antigens from pathogenic microorganisms [4, 5]. Therefore, intestinal epithelial cells (IECs), located in the interface between the intestinal lumen, inhabited by a multitude of microorganisms, and the lamina propria, densely populated by immune cells, assume a privileged position, playing a daunting task of segregating, physically and biochemically, these such diverse environments, but also of integrating signals between the microbiota and the host immune system [1, 5, 6]. Thus, in order to preserve the intestinal homeostasis, a friendly and balanced interaction between the commensal flora, the intestinal epithelium and the local immune cells is continuously required [7]. A breakdown in this dynamic cooperation can result in intestinal inflammation, typically present in a well-known pathology, the Inflammatory Bowel Disease [7].

2. Inflammatory Bowel Disease

2.1 Overview

The Inflammatory Bowel Disease (IBD) is a group of idiopathic and chronic inflammatory disorders of the gastrointestinal tract, encompassing, as main forms, the Crohn's Disease (CD) and the Ulcerative Colitis (UC) [8]. Up to this date, IBD does not have cure and it is a lifelong disorder, whose clinical course is characterized by alternating periods of relapses and remissions. Therefore, this disease accounts for a huge economic impact to the healthcare systems, associated with the constant need for visits to physicians, frequent hospitalizations and palliative drugs. On the other hand, it involves indirect costs with great impact in the economy of the countries, related to the decrease in patient quality of life and the consequent loss of productivity or increase in absenteeism [8-11]. This becomes even more impressive if we take into account that IBD affects normally people during their economically productive adult age (age peak for CD is 20-30 and for UC is 30-40) [12].

IBD is estimated to have the highest incidence and prevalence rates in industrialized countries, mainly in countries of North Europe and of United States of America [11]. In agreement with the World Gastroenterology Organisation Global Guidelines of 2010 [13], those rates are rising in areas with previous low-incidence, namely in countries of Eastern Europe and Asia, for reasons not fully determined.

Until now, the etiology of IBD remains uncertain, but it is believed that IBD is a multifactorial disorder, in which the interplay between a genetic predisposition and environmental factors, such as stress, diet and antibiotic intake, induces an aberrant immune reaction to commensal microorganisms, culminating in the perpetuation of uncontrolled and exacerbated inflammatory and oxidative responses, which lead to serious intestinal injury [2, 7, 12, 14, 15].

Although the main conditions of IBD (CD and UC) have some overlapping characteristics, the inflammation in UC is diffuse and typically restricted to the mucosa of the colon, whereas, in CD, it is focal, transmural and sporadically granulomatous in the ileo-cecal or ileo-colic areas, and may affect the whole gastrointestinal tract, particularly the ileum and the colon [9, 10]. The major symptoms of IBD include diarrhoea, tenesmus, abdominal pain, nausea, vomiting, fever, weight loss and fatigue, varying from mild to severe in periods of relapses and even vanishing in periods of remissions [13, 16]. Moreover, IBD patients can suffer not only

from intestinal complications, including bowel perforation and fistulas, toxic megacolon and even, in most severe cases, colorectal cancer, but also from extra-intestinal complications, affecting a multitude of organs, such as the skin, the liver and the kidney [9, 13, 16, 17]. Therefore, the complexity of intestinal and extra-intestinal manifestations with insidious beginning in these patients and the appearance of symptoms frequently shared with other gastrointestinal pathologies, make the diagnosis of IBD a difficult task even for a trained specialist [9]. Thus, the accurate diagnosis of IBD implies taking into account the medical history and the clinic evaluation of the patient, together with laboratory, endoscopic, histologic and radiologic findings [8, 18].

2.2 Molecular mechanisms underlying Inflammatory Bowel Disease

Although the molecular mechanisms underlying IBD are still uncertain, it is known that some of them are related to dysfunctions in the structure and in the function of the intestinal epithelium as an anatomical barrier (section 2.2.1) and others are correlated to the loss of intestinal epithelium ability to properly modulate the interactions between intestinal microbiota and the immune system (section 2.2.2) [5, 12].

2.2.1 Intestinal epithelial barrier and its dysfunction in Inflammatory Bowel Disease

"Good fences make good neighbours", by Robert Frost (1914)

The intestinal epithelium, the largest of the mucosal surfaces of the human body, is composed by a single-layer of columnar cells, organized into crypts and villi, which are continuously renewed by pluripotent intestinal epithelial stem cells, present in the base of intestinal crypts [1]. Pluripotent stem cells give rise to several subtypes of specialized mature epithelial cells (**Figure 1**), including enterocytes, goblet cells, Paneth cells and enteroendocrine cells [19, 20].

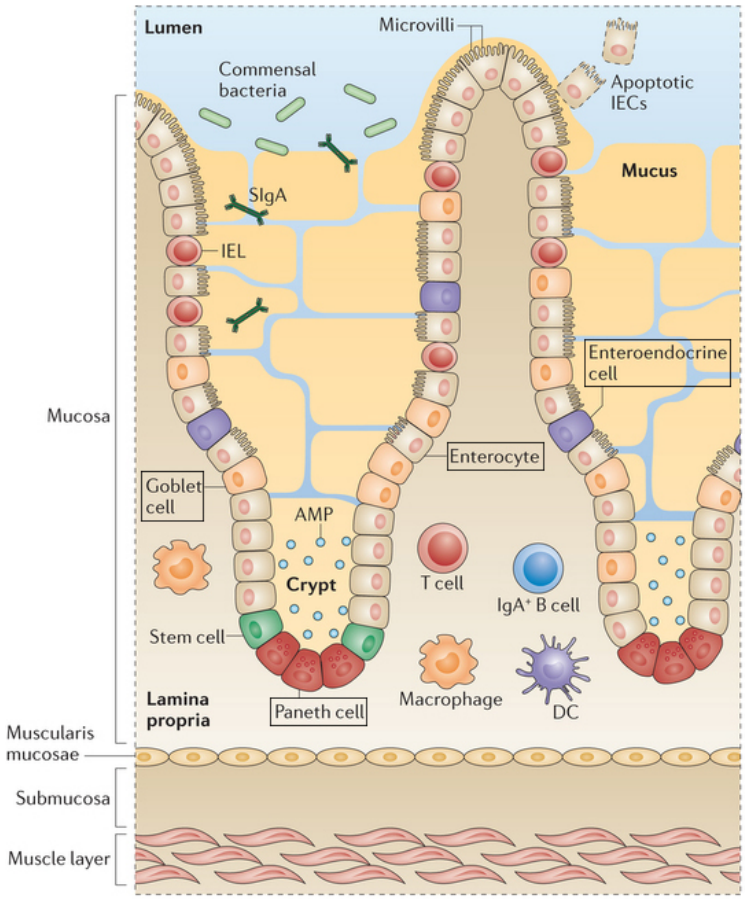


Figure 1 - Illustration representing some specialized intestinal epithelial cells existing in jejunum and its organization into crypts and villi (reproduced with permission from [19]).

Being located between luminal microorganisms (in the apical side) and immune cells (in the basolateral side), the intestinal epithelial barrier has to resort to some strategies to keep these environments apart. This is accomplished thanks to the existence of the apical junctional complex along with desmosomes, to the production of mucus and antimicrobial peptides and also to the secretion of IgA, all of which reinforce the intestinal epithelial barrier, either physical or biochemically, as discussed below [3, 21]. Therefore, a breakdown in the mechanisms that normally minimize the contact between the luminal bacteria and the intestinal mucosal surface may contribute to the development of IBD [3].

A - Junctional complex

IECs are structurally and functionally polarized cells, with an apical surface facing the luminal milieu and a basolateral surface facing the basement membrane and the lamina propria [22]. Under normal conditions, the paracellular space between IECs is sealed by the apical junctional complex (**Figure 2**), which is formed by the tight junction (TJ) and the subjacent adherens junction [21]. Tight junctions are composed by transmembrane proteins, such as those belonging to claudin family, scaffolding proteins and regulatory molecules, including kinases, and they are considered of great importance in the regulation of TJ permeability [21, 23]. Adherens junctions are formed by a family of transmembrane proteins called cadherins, and together with desmosomes (**Figure 2**), reinforce the cellular proximity between IECs [21]. Numerous studies have reported changes in the expression and structure of several intestinal TJ proteins in IBD patients [22, 23]. However, until now, it is not clear whether the impairment of the transmembrane proteins that integrate the junctions is a cause or a consequence of intestinal inflammation [12].

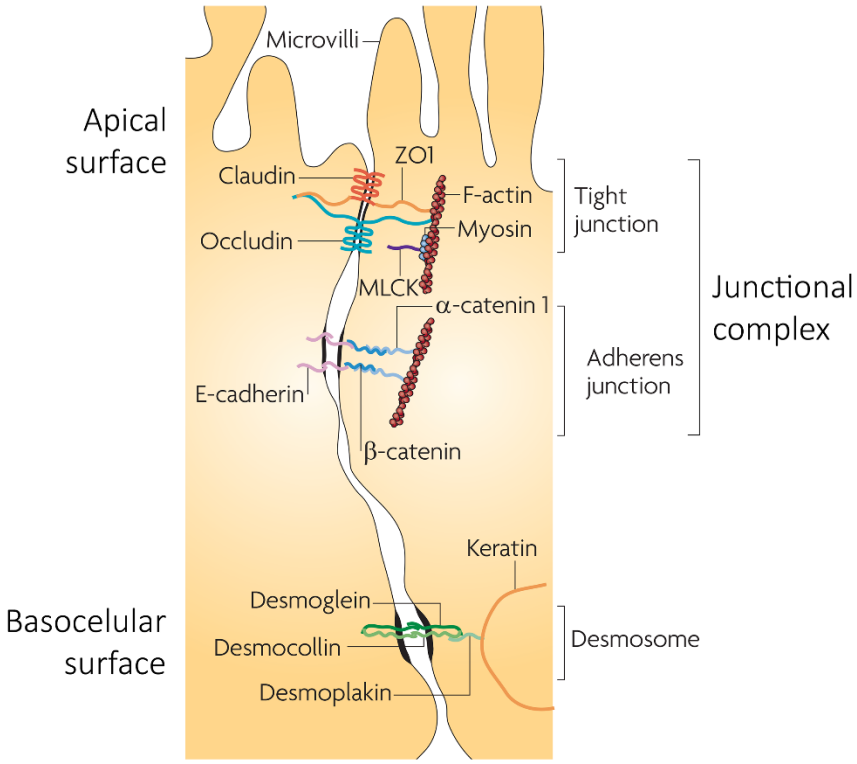


Figure 2 - Schematic illustration showing the junctional complex and the desmosome of an intestinal epithelial cell (modified from [21]).

B - Mucus layer

The intestinal epithelium is covered by a mucus layer (**Figure 3**), mainly composed by mucins (highly glycosylated proteins) and by other molecules, such as trefoil peptides, which are produced by a specific type of epithelial cells, the goblet cells [24]. Until now, 20 different mucins (MUCs) have been identified, being classified as secretory or membrane-associated mucins. MUC2, the first recognized human secretory mucin, is considered the major secretory intestinal mucin [24].

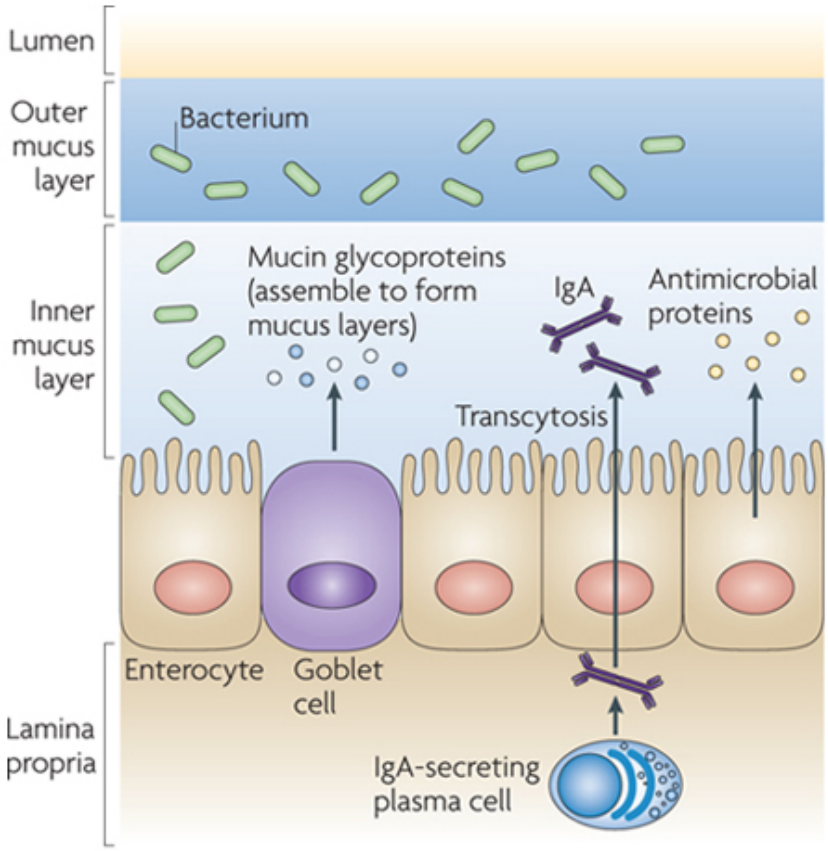


Figure 3 - Schematic drawing representing some mechanisms used by IECs to prevent the invasion of bacteria (reproduced with permission from [3]).

Goblet cells are present in a greater amount in the distal colon than in the upper small intestine, which seems to be correlated with the increasing number of microorganisms harboured throughout the intestine (10^3 to 10^8 organisms per gram of luminal contents in the

small intestine and 10^{10} to 10^{12} organisms per gram in the colon) [2, 19]. Therefore, contrasting with small intestine, where the mucus layer is thinner and discontinuous, in the large colon the mucus lining is stratified in two sheets: the inner, denser and therefore more resistant to microbial invasion, and the outer, looser and highly populated by microorganisms [2, 4, 19].

The importance of a proper mucus layer becomes evident in mice deficient in *MUC2*, which spontaneously develop colitis [2]. Further, more evidence demonstrates that colon tissue obtained from CD patients has a disturbed mRNA expression of MUC2 and of MUC12, being the latter also decreased in colonic samples from UC patients [25]. On the other hand, it has been reported that impairments in mucin glycosylation can also lead to defects in mucus barrier, enhancing the susceptibility to experimentally induced colitis observed in mice [24, 26]. Therefore, it has been suggested that alterations either in mucin production or in mucin glycosylation can be related to the pathophysiology of IBD [24].

C - Antimicrobial peptides

Beyond the mucus layer, an extra immune mechanism that reduces the contact between the luminal microorganisms and the IECs involves the secretion of antimicrobial peptides (AMPs), namely defensins, cathelicidins, lactotransferrin and lysozyme, by several IECs, such as enterocytes/colonocytes, goblet cells and Paneth cells [3, 27]. The AMPs become trapped in the mucus layer, acting as a biochemical barrier [4], as depicted in **Figure 3**. The majority of AMPs are capable of killing bacteria by enzymatic attack on the cell wall or by destabilizing the inner membrane of Gram-negative bacteria [3].

Some recent research found that impairment of beta-defensin 2 expression predisposed individuals to colonic CD [28], which suggests that the role of defensin peptides should not be undervalued in the context of IBD.

D - Immunoglobulin A

The accumulation of Immunoglobulin A (IgA) in the intestinal lumen (**Figure 3**) is another mechanism of protection used by intestinal epithelial barrier in order to prevent the invasion of luminal bacteria either by trapping them in the mucus layer or by inducing the phagocytic clearance of microorganisms that penetrate the intestinal barrier [1, 3, 4]. IgA is secreted by plasma cells in the lamina propria, as dimeric IgA, and transcytosed into the intestinal lumen

through IECs [3]. The process of IgA transcytosis is mediated by a glycoprotein, the polymeric immunoglobulin receptor (pIgR), which undergoes an endoproteolytic cleavage near the plasma membrane of IECs, allowing the release of its secretory component into the lumen together with IgA, forming the secretory IgA complex. In IBD, it has been suggested that, for some reason not fully understood, the expression of pIgR is downregulated, the transcytosis of dimeric IgA is reduced and IgA seems to be accumulated in the lamina propria. All of these defects can impair IgA trafficking and its accumulation in the intestinal lumen, contributing to IBD pathogenesis [29].

On the other hand, according to Macpherson and collaborators [30], patients with either form of IBD, in the active phase, produce high levels of IgG and low levels of secretory IgA. The authors pointed that the high production of IgG, towards proteins existent in the cytoplasm of commensal bacteria, was related to the episodes of relapses, observed in those patients. In line with this, some authors suggest that the shift from the secretion of protective subtype antibodies (IgA) to aggressive subtype antibodies (IgG) can be subjacent to the development of the mucosal inflammation in IBD patients [31].

E - Pattern Recognition Receptors

Despite the previously described mechanisms employed by intestinal epithelium to insulate bacteria from the intestinal surface, microbe-associated molecular patterns (MAMPs) can be detected by IECs or by immune cells, through evolutionarily conserved receptors, termed pattern-recognition receptors (PRRs), namely toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) [2, 6].

E.1 Toll-Like Receptors

TLRs are a class of transmembrane glycoproteins constitutively or inducibly expressed by different cell types in the intestinal tract, namely IECs, macrophages, dendritic cells and $CD4^+$ T cells. These receptors are capable of recognizing multiple MAMPs, such as lipopolysaccharide (LPS) and flagellin, and can be grouped according to their localization either on the cell surface (TLR1, 2, 4, 5, 6) or in intracellular compartments (TLR3, 7, 8, 9), such as endosomes and lysosomes [32-34]. TLRs are conjugated proteins formed by an extracellular or luminal ligand-binding domain containing leucine-rich repeat motifs and a cytoplasmic

Toll-interleukin-1 receptor homology (TIR) domain [32, 34]. The TIR domain is crucial for the interaction between homo and heterodimeric TLR subunits and also for the anchorage of cytoplasmic adaptor molecules, including myeloid differentiation primary response 88 protein (MyD88), TIR domain-containing adaptor protein inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) [32, 35]. Activation of TLRs generally culminates in the activation of a well-known signalling cascade, the nuclear factor-kB (NF-kB), to elicit a pro-inflammatory response, attempting to eradicate the potential pathogens [2, 11, 12]. Conversely, in the absence of pathogenic agents, accelerating research has demonstrated that the intestinal epithelium takes advantage of signals derived from commensals, through TLR activation, to strengthen its function as a barrier, for instance, by increasing the production of mucus or the transport of IgA into the lumen, and also to maintain the tolerance of immune cells against microbiota (as it will be discussed below, in 2.2.2-D section) [1, 2, 11]. As highlighted above, although the intestine must preserve the reception of signals derived from commensals to maintain the intestinal homeostasis, the intestine has also to develop strategies to avoid the hyper-stimulation of pro-inflammatory signalling pathways, namely NF-kB, towards commensals [1, 6, 36]. Therefore, in normal conditions, TLRs are positioned preferentially on the basolateral surface of IECs as well as in intracellular compartments in detriment of IEC apical surface, decreasing their accessibility by commensals, and, on the other hand, the commensal bacteria by themselves can inhibit NF-kB activation. This is accomplished, for example, by inhibiting the degradation of the inhibitor of kappa B (IkB) and thereby preventing the entry of NF-kB in the nucleus or by inducing the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), which is also capable of inhibiting NF-kB pathway, as referred below on section 2.3.5 [1, 2, 5, 6]. However, in the case of IBD, IECs display higher expression of some TLRs at their apical side than in physiological conditions, thus increasing the possible interaction with commensal microorganisms [32, 37, 38]. Accordingly, it seems possible that IECs upregulate the expression of TLRs during intestinal inflammation upon stimulation with cytokines, such as TNF-α and IFN-γ [32, 37]. The sustained dysregulation of TLR signalling and the consequent over-activation of the pro-inflammatory NF-kB cascade are believed to contribute to chronic intestinal inflammation and so to IBD pathogenesis [7, 12, 32].

E.2 Nucleotide Oligomerization Domain (NOD)-Like Receptors

NLRs are cytoplasmic proteins, structurally formed by an N-terminal effector domain (usually a caspase recruitment domain, CARD), a central conserved nucleotide-binding oligomerization domain (NOD) and a carboxy-terminal leucine-rich repeats (LRRs) [35, 38]. NOD1 and NOD2 were the first NLRs to be reported and they are activated by different peptidoglycan motifs, D-γ-glutamyl-meso-diaminopimelic acid and muramyl dipeptide, respectively [35]. While NOD1 is ubiquitously expressed in human cells, NOD2 is mainly expressed in epithelial cells, phagocytes and dendritic cells [38]. Both NOD1 and NOD2 expressions are normally low in intestinal epithelial cells, but can be upregulated by inflammatory stimuli, such as IFN-γ [11, 38]. Upon activation, NOD1 and NOD2 lead to the activation of NF-kB and mitogen-activated protein kinases (MAPKs), resulting in the transcription of several pro-inflammatory genes [12, 39].

NOD2 was the first gene to be connected to IBD and it is well-accepted that polymorphisms in *NOD2* are strongly related to CD [40-42]. Although, there are several possible explanations for the involvement of *NOD2* mutations in the pathogenesis of CD, one of the most debated hypothesis suggests that the absence of NOD2 expression by Paneth cells results in a defective production of AMPs, allowing the proliferation of bacteria in the intestinal crypts and making the intestinal barrier and the intestinal milieu more exposed to the stimulation by antigens [17, 43], as illustrated in **Figure 4**.

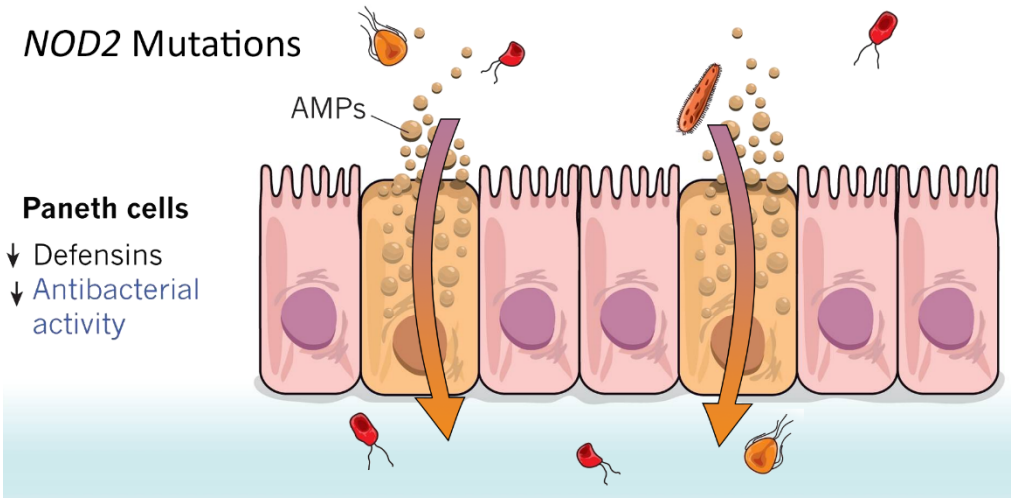


Figure 4 - Schematic representation illustrating the possible contribution of *NOD2* mutations in the pathogenesis of CD (adapted from [42]).

2.2.2 Host immune responses and their dysregulation in Inflammatory Bowel Disease

The intestinal immune system, often referred to as gut-associated lymphoid tissue (GALT), is a particular component of the immune system, which plays a crucial role in promoting immune responses against potential pathogens as well as in promoting tolerance against dietary antigens or commensal microorganisms [44].

A - Gut-Associated Lymphoid Tissue

GALT is divided into two different types of compartments, the inductive and the effector compartments [45], as shown in **Figure 5**. The inductive compartments are organized lymphoid tissues, such as Payer's patches (PPs; located in the small intestine and particularly abundant in the distal ileum), colonic patches (located in the colon and rectum), mesenteric lymph nodes (MLNs) and isolated lymphoid follicles (ILFs). The effector compartments include the lymphocytes present throughout the epithelium and the lamina propria [19, 45].

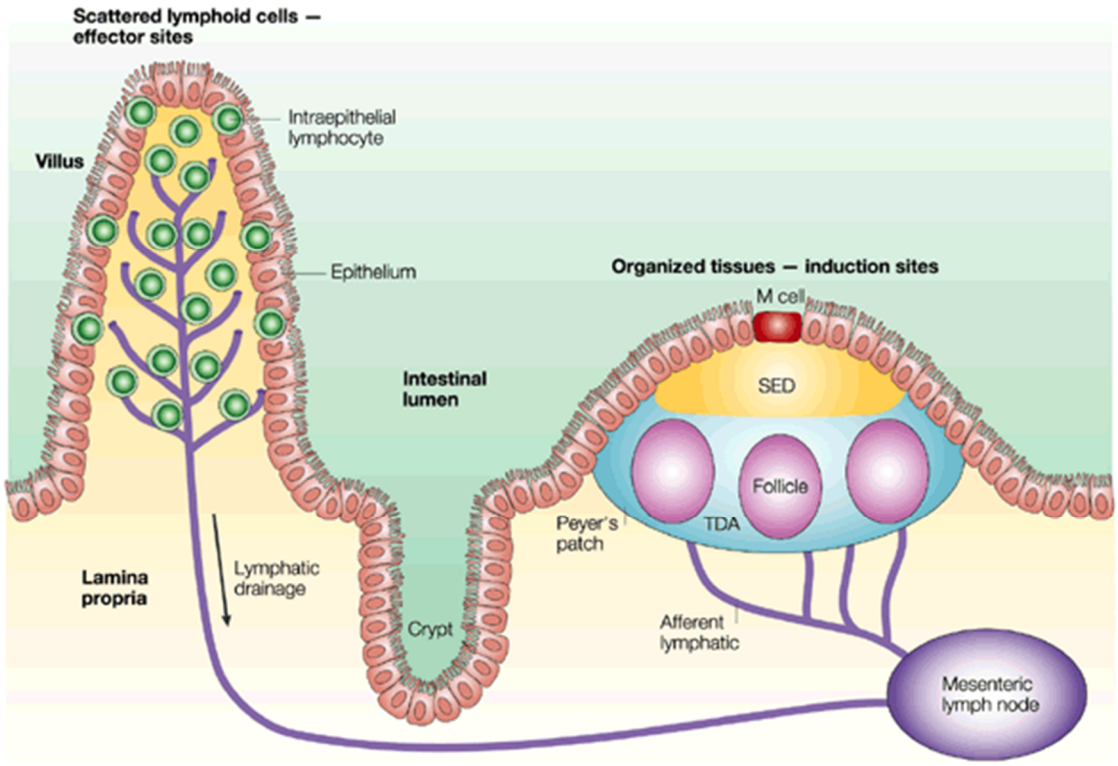


Figure 5 - Illustration of the gut-associated lymphoid tissue. SED, subepithelial dome; TDA, thymus-dependent area (reproduced with permission from [45]).

B - Uptake of luminal antigens and their presentation to immune cells

Mature PPs comprise B-cell follicles and T-cell areas, which are separated from the intestinal lumen, thanks to the follicle-associated epithelium (FAE) and the subepithelial dome (SED). The most important characteristic of FAE is the existence of Microfold (M) cells [6, 45]. These cells are specialized epithelial cells, which have a reduced mucus layer and a modified apical and basolateral surfaces, making them able to capture antigens and deliver them to professional antigen-presenting cells (APCs), mainly dendritic cells (DCs), in the epithelium or in the SED [6, 46]. M cells are not exclusive of PPs and they can also be present over ILFs in the colon [19]. Although M-cell-mediated-transcytosis is a common route of antigens uptake (**Figure 6B**), there are also other possible routes used by enterocytes and by mononuclear phagocytes to uptake luminal antigens.

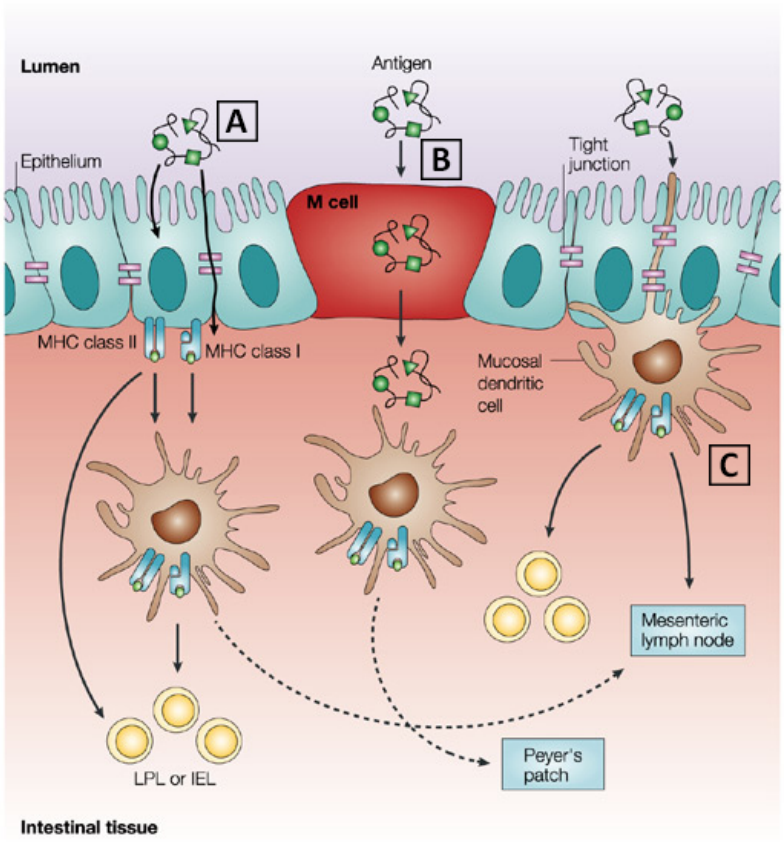


Figure 6 - Some routes of antigen uptake at the intestinal level. A) Epithelial cells capture antigens and present processed antigens to intraepithelial lymphocytes (IEL) or to lamina propria lymphocytes (LPL) or transfer already processed antigens to dendritic cells. B) M cells uptake antigens and deliver them to dendritic cells. C) Dendritic cells uptake antigens through the extension of their finger-like projections (reproduced with permission from [46]).

In fact, it is known that gut epithelial cells can also mediate active endocytosis and process and present antigens bound to major histocompatibility complex (MHC) class I or class II molecules to intraepithelial lymphocytes or lamina propria lymphocytes (**Figure 6A**), but, apparently, they are not able to prime naïve T cells, due to the lack of co-stimulatory molecules expression [6, 46]. Although the significance of these data awaits further investigations, it seems that through that way, IECs contribute to T cell non-responsiveness and consequently to natural tolerance [6, 47, 48]. Further, IECs can also transfer already processed antigens to DCs (**Figure 6A**) [46]. DCs are mononuclear phagocytes, which have a major importance in the initiation and the development of intestinal immunity against pathogens and in the promotion of tolerance against commensal microorganisms [49]. These professional APCs have been reported throughout the intestine, in PPs, in ILFs and in MLNs, but they can also be found in the lamina propria and in association with the villous epithelium [50]. Until now, the mechanisms underlying the ability of DCs to acquire directly antigens have remained uncertain. However, some recent studies suggest that DCs, positioned inside the intestinal epithelium, can capture luminal antigens through the extension of their finger-like projections (**Figure 6C**) [51]. Another possible mechanism used by DCs to uptake antigens is through interaction with antigen-channeling goblet cells [49, 51]. In secondary lymphoid organs (PPs and MLNs), antigen-loaded DCs present the antigens to adaptive immune cells [3, 52]. Beyond DCs, macrophages are another type of mononuclear phagocytes with great abundance in the intestinal lamina propria. Macrophages can also sample luminal antigens directly, extending their trans-epithelial dendrites (TEDs) [49, 51]. In contrast to DCs, macrophages remain in close contact with IECs, acting as highly effective phagocytes to promote the clearance of antigens [52].

C - General adaptive immune dysfunctions in Inflammatory Bowel Disease - the classical point of view in confrontation with recent findings

The majority of studies indicate that chronic intestinal inflammation, observed in patients with IBD, is essentially related to the intensive recruitment and inappropriate retention of mucosal activated $CD4^+$ T-helper (Th) lymphocytes, which proliferate and differentiate from naïve $CD4^+$ Th cells into several subtypes of T-cells, including Th1 and Th2 cells [2, 40, 53].

Until a few years ago, it was argued that CD pathogenesis was related to an exaggerated Th1 response, whereas UC pathogenesis was related to an exaggerated Th2 response [53, 54]. While

Th1 cells can mediate immunity against intracellular pathogens, producing pro-inflammatory cytokines, such as TNF-α and IFN-γ, which, in turn, among other mechanisms, promote the release of further large amounts of pro-inflammatory cytokines from macrophages, Th2 cells can mediate immunity against extracellular pathogens, secreting cytokines, such as IL-4, IL-5 and IL-13, which induce B cell activation and promote antibodies production [55]. Further, Th1 and Th2 cells can cross-regulate each other and, thus, cytokines produced by Th1 inhibit the differentiation of naïve T cells into Th2 cells and vice-versa [55].

The Th1/Th2 dichotomy was greatly supported by intestinal tissue sections collected from patients with active CD, demonstrating increased levels of Th1-polarizing cytokines (namely IL-12) in comparison with normal tissues and UC patients [53]. However, evidence gathered over the last years has demonstrated that the Th1/Th2 paradigm claimed for IBD was too simplistic and the overlapping cytokine profile underlying CD and UC justified the need to update that concept [53, 56]. In fact, the recognition, in more recent years, of another subpopulation of T lymphocytes, Th17 cells, has reinforced the requirement of changing the traditional Th1/Th2 paradigm [2, 54]. Th17 cells, present in the intestine, mainly in the terminal ileum, after being stimulated by IL-6, TGF-β and IL-23, following the activation of dendritic cells and/or activated epithelial cells, are able to secrete pro-inflammatory cytokines, namely IL-17 and IL-21, which lead to the recruitment of other cells, such as neutrophils [20]. Many studies have demonstrated that the production of Th17-related cytokines are increased in intestinal biopsies and in lamina propria mononuclear cells of patients with CD and UC, being possibly implicated in the pathogenesis of both forms of IBD, but particularly in CD [2, 53, 54, 57].

On the other hand, although UC was initially related to a Th2 profile, the levels of IL-4, a classical Th2 cytokine, did not show to be augmented in UC patients, which suggested that UC patients do not show all of the features of classical Th2 cells [17, 57]. Actually, it has been suggested that UC patients present an atypical Th2 profile, characterized by the existence of elevated amounts of IL-13 secreted by CD1d-restricted non-classical natural killer T cells (NKT cells) [14, 20, 56-58]. Accordingly, in a mouse model for UC, it was demonstrated that the suppression of IL-13, produced by NKT cells, could prevent colitis development [17, 57]. However, some contradictory results have emerged in more recent studies, since it has been reported that patients with UC did not show high levels of IL-13. Altogether it becomes quite clear that further work is mandatory in this field to better elucidate this issue [57]. Furthermore, it has been reported a disturbed balance between the number of regulatory T cells (Treg cells)

and of effector T cells in active IBD, existing a preferential differentiation of naïve T cells into effector T cells, instead of into Treg cells, in those patients [11]. Treg cells produce anti-inflammatory cytokines, such as IL-10 and TGF-β, and they show to be central players in suppressing immune responses, ensuring tolerance towards commensal microorganisms [57].

D - Intestinal epithelial cells in the orchestration of host immune responses

Contrary to what was initially believed, IECs may be not merely important to segregate the intestinal lumen from the intestinal milieu, but may be also crucial to modulate the function of host immune cells, for instance, through the secretion of cytokines and chemokines, which interfere directly or indirectly with the function of antigen-presenting cells and, thereafter, with the subsequent cellular and humoral adaptive immune responses [1, 5, 6, 42]. Although this theme is still in its infancy, several authors are increasingly interested in studying the influence of IECs over the host immune responses and how the dysregulation of this finely-tuned balance can be implicated in the IBD pathogenesis. Some examples of the possible influence of IECs over the host immune regulation are discussed below, either in physiological conditions or in IBD.

D.1 Immune regulation in physiological conditions

As shown in **Figure 7A,** after recognition of commensal bacteria via PRR signalling, IECs secrete, for instance, thymic stromal lymphopoietin (TSLP), transforming growth factor-β (TGF-β) and retinoic acid (RA), which convert DCs and macrophages into a tolerogenic phenotype [1, 5, 6, 59]. Therefore, IECs-conditioned DCs induce the differentiation of naïve $CD4^+$ T-cells into regulatory T cells (Treg), by a TGF-β and RA-dependent mechanism, induce the maturation of B cells into IgA-secreting plasma cells and promote the imprinting of gut-homing phenotype on primed T-cells and B-cells [1, 2, 5, 60].

On the other hand, macrophages, conditioned by IEC-derived TSLP and by contact-dependent interaction with IEC-expressed semaphoring 7A (SEMA7A), produce IL-10, leading to the expansion in number of Treg cells **Figure 7C** [1, 5, 7].

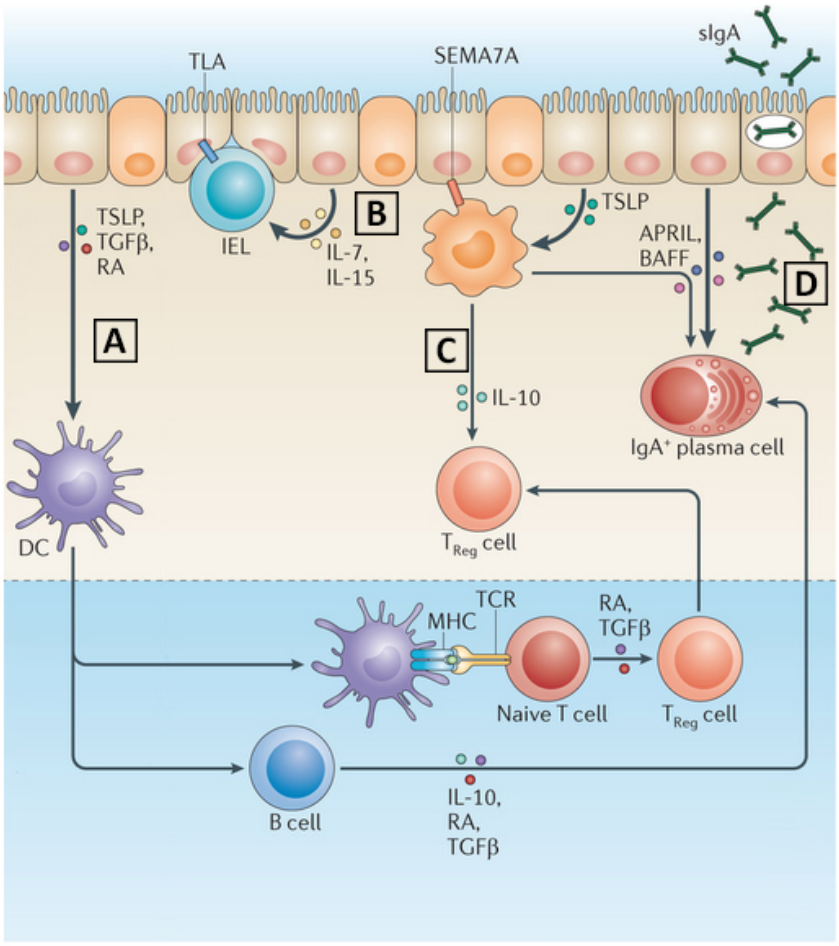


Figure 7 - Representative illustration showing some examples of the orchestration of host immune responses by intestinal epithelial cells in physiological conditions (reproduced with permission from [1]).

Furthermore, IECs can also release a proliferation-inducing ligand (APRIL) and a B-cell activating factor (BAFF), which induce IgA class switching and the production of IgA by B cells (**Figure 7D**) [1, 6, 7, 60]. Furthermore, IEC-derived TSLP induces the production of APRIL and BAFF by macrophages, increasing the stimulation of B cells (**Figure 7D**) [1].

Also, it has been suggested that IECs-derived signals, including IL-7 and IL-15, regulate the recruitment, the maturation and even the function of intraepithelial lymphocytes (IELs) (**Figure 7B**). IELs are a specific subset of T cells, which are localized between IECs, establishing intimate contact with those cells, and whose development and function are not fully clarified [1, 5].

Moreover, it is known that IECs can still regulate innate lymphoid cells (ILCs). ILCs are a recently identified population of innate immune cells present in the intestine, which revealed to be important to the maintenance of intestinal homeostasis, but whose involvement in the

development of the inflammatory process is not fully understood [1, 4, 7]. According to their developmental requirements and different patterns of cytokine expression, ILCs are divided into three groups that present functional similarities with $CD4^+$ Th1, Th2 and Th17 cells [1]. However, in spite of being important sources of cytokines, they lack the main features of Th immune cells, including recombined antigen-specific receptors [1, 4, 20, 57]. There are some examples of the regulation of ILCs by IECs. For instance, it has been described that IEC-derived TSLP, IL-25 and IL-33 can stimulate the group 2 of ILCs. However, IL-25 produced by IECs is also capable of suppressing the function of the group 1 and 3 of ILCs, by preventing, for instance, the production of pro-inflammatory cytokines by intestinal macrophages [1, 7, 20].

D.2 Immune regulation in Inflammatory Bowel Disease

A decreased production of TSLP by IECs has been described in CD patients, which makes these cells incapable of polarizing DCs into a tolerogenic phenotype [54]. Accordingly, some evidence suggests that in IBD patients, DCs promote the differentiation of naïve $CD4^+$ T-cells into Th1 and Th17 cells, instead of into Treg cells, promoting an abnormal pro-inflammatory response against commensals [11]. Furthermore, some data demonstrate that both intestinal DCs and macrophages, present in the lamina propria of IBD patients, produce elevated levels of pro-inflammatory cytokines, as compared to cells of healthy individuals, which is also in line with the dysregulation of the tolerance against commensals [39, 52]. Also, T cells from IBD patients appear to be refractory to TGF-β, which may contribute to the dysregulation of host immune responses, and consequently to the perpetuation of chronic intestinal inflammation [7, 17, 20].

E - Gathering thoughts

Taking the above observations together, it is possible to infer that, in physiological conditions, IECs have an active role in the maintenance of an environment propitious to a peaceful interaction with the commensal bacteria, however, they may be also pivotal to the breaking down of the normal tolerance towards commensals, resulting in intestinal disease [42, 47]. This can be understood considering not only the possible impairment in the functions of the intestinal epithelium as an anatomical barrier, making it vulnerable to the invasion by commensal bacteria and by pathogenic agents, but also in light of the decreased production of

tolerogenic mediators and/or increased production of pro-inflammatory mediators, which, all together, contribute to the perpetuation of the intestinal inflammation and oxidative stress, described in IBD patients [11, 54]. However, although much is known about the interplay between commensals and IECs and their contribution to the alteration of IEC function, involving, for instance, the increase in the production of mucus and AMPs, knowledge on the mechanisms underlying the control of host immune responses by IECs is scarce, being this, perhaps, a reason for several authors underappreciate the actual value of IECs in the IBD context [5].

2.3 Cellular signalling cascades of great relevance in Intestinal Inflammation

2.3.1 Nuclear Factor-kB (NF-kB) pathway

The nuclear factor-kB (NF-kB) is one of the most studied transcription factors, detected in both immune and non-immune cells, involved in the regulation of several important physiological events, including inflammation, oxidative stress, apoptosis, survival and immune responses [61-65].

In mammals, NF-kB is composed of five different subunits organized into homo and heterodimers: p65/RelA, c-Rel and RelB and the precursor proteins p105 and p100, processed into p50 and p52, respectively [62]. The most common activated dimer of NF-kB is formed by p65 with p50 [66]. In resting cells, NF-kB dimers reside predominantly in the cytoplasm, in an inactive form, sequestered by inhibitory proteins of the IkB family, namely IkB-α, IkB-β and IkB-ε [64, 66].

Two different NF-kB-activation pathways have been described, the canonical/classical and the alternative/non-classical pathway [61]. However, the classical NF-kB pathway is the most frequently observed, being activated by a large number of stimuli, including pro-inflammatory cytokines, namely IL-1 and TNF-α, microbial products, such as LPS, and oxidative stress [62, 64, 66-69]. In general terms, the NF-kB-activators stimuli converge to the activation (phosphorylation) of the IkB kinase (IKK) complex, constituted by IKKα/IKK1 and by IKKβ/IKK2 as catalytic subunits and by IKKγ/NEMO as the regulatory subunit. Once activated, the IKK complex (mainly the IKKβ subunit) phosphorylates IkB-α on 32 and 36 serine residues, which is then ubiquitinated and degraded via proteasomal degradation, allowing the translocation of free NF-kB dimers into the nucleus [64, 66, 70]. Growing evidence suggests

that post-translational modifications of NF-kB components are required for the full transcriptional activity of NF-kB, namely phosphorylation and acetylation [62, 64, 66]. Activated NF-kB binds to DNA kB sites and regulates the expression of several genes, such as genes encoding pro-inflammatory enzymes (inducible nitric oxide synthase, iNOS; cyclooxygenase-2, COX-2) and pro-inflammatory cytokines or chemokines (IL-1, TNF-α, IL-8, IL-6, IL-12) which, in turn, may lead to the recruitment of immune cells, with the subsequent production of further pro-inflammatory mediators, perpetuating the inflammatory response and leading ultimately to intestinal injury (**Figure 8**) [65-68].

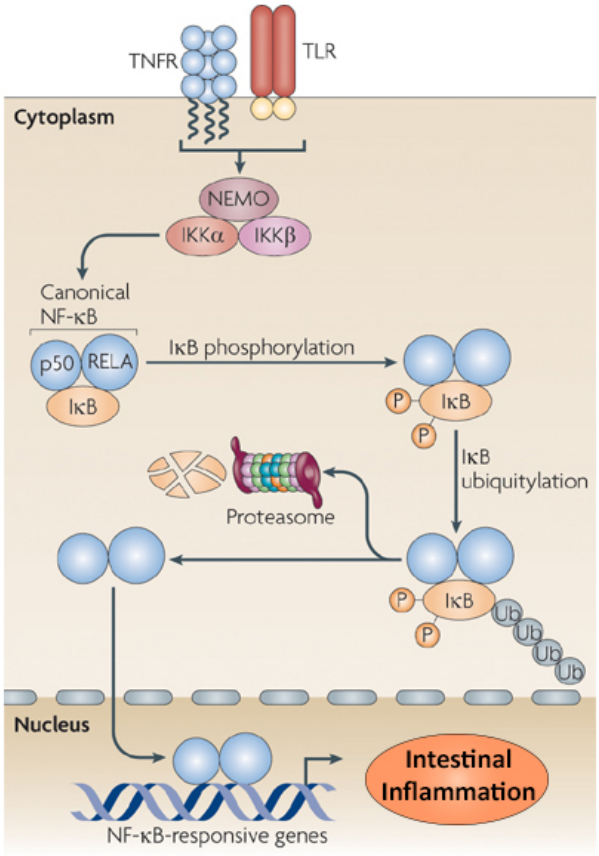


Figure 8 - Schematic representation showing that NF-kB activation promotes the transcription of pro-inflammatory genes and maintains active intestinal inflammation (adapted from [63]).

In fact, although NF-kB pathway is truly important to maintain the intestinal homeostasis (as already referred in previous sections), chronic hyper-activation of this pathway contributes to chronic intestinal inflammation and therefore to IBD pathogenesis [12, 61, 65, 71]. Consistently, some reports demonstrate that NF-kB is highly activated in IECs and also in

macrophages isolated from the inflamed mucosa of patients with IBD [63, 66, 72-74]. Also, evidence shows that NF-kB up-regulation can be closely related to the severity of intestinal inflammation [73]. For this reason, the inhibition of this potent pro-inflammatory signalling cascade has been widely investigated to control inflammatory diseases, as IBD, with successful results already verified in several animal models [63, 66, 73]. Furthermore, some drugs currently used for the treatment of IBD patients, such as anti-TNF-α monoclonal antibodies and corticosteroids, are able to inhibit NF-kB pathway, further supporting the relevance of this pathway in the context of intestinal inflammation [66, 73].

2.3.2 Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway is another relevant signalling cascade in the IBD context, playing an important role in several cellular processes, namely cell growth, differentiation, proliferation, apoptosis and inflammation [75, 76].

In mammals, there are four types of JAKs, (JAK1, JAK2, JAK3 and TYK2) and seven types of STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6), which can form homo or heterodimers [77]. The major activators of JAK/STAT pathway are cytokines, mainly interferons and interleukins, and growth factors [75, 78]. Given the crucial role of IFN-γ in the pathogenesis of IBD [79] and the recognition that IFN-γ biological responses are primarily mediated by JAK/STAT pathway [80], the involvement of this signalling cascade has been investigated in the context of IBD.

It is known that the binding of IFN-γ to its cell-surface receptor (IFN-γ receptor, IFNGR) leads to the activation of JAK1 and JAK2 through auto- and/or trans-phosphorylation [81]. Activated JAKs phosphorylate IFNGR, providing a docking-site for the recruitment of STAT1 [78, 81, 82]. JAKs also have the ability to phosphorylate STAT1 on its tyrosine (Tyr) residue, at position 701, which makes STAT1 ready to homodimerize and to translocate into the nucleus. There, it binds to specific DNA consensus sequences to initiate the transcription of STAT-responsive genes, such as genes encoding pro-inflammatory enzymes (e.g. iNOS), pro-oxidant enzymes (e.g. NADPH oxidase 1) and chemokines (e.g. CXCL9, CXCL10 and CXCL11) (**Figure 9**) [82-88].

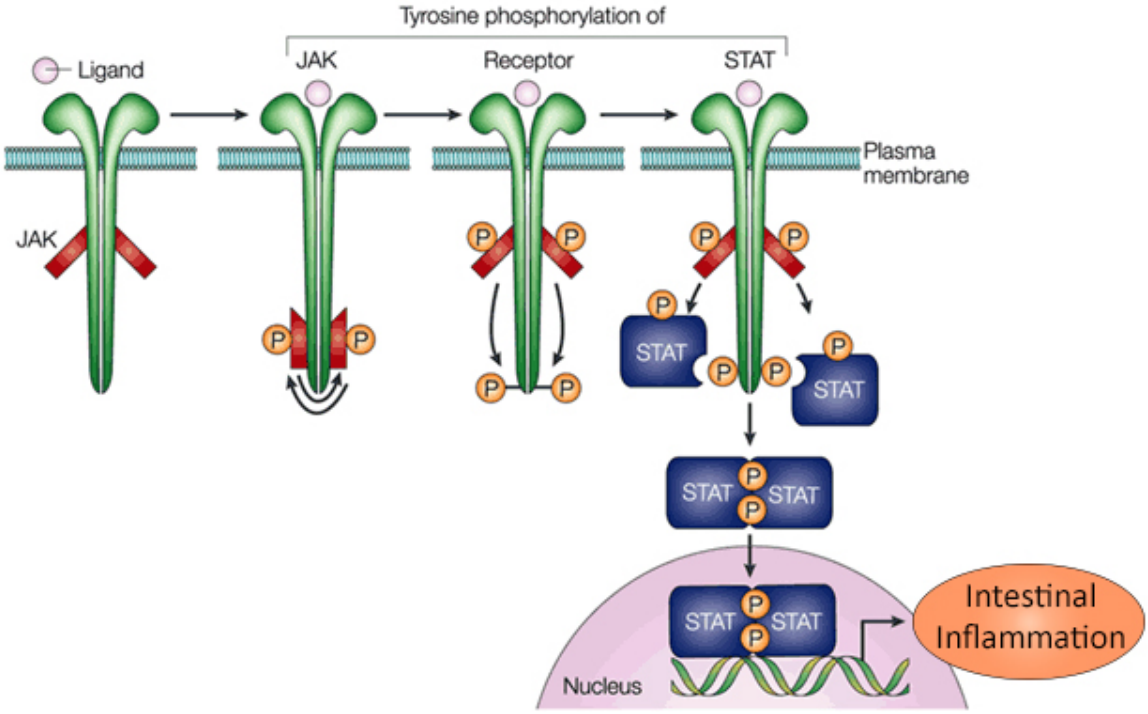


Figure 9 - Schematic picture illustrating the activation of JAK/STAT pathway and the subsequent enhancement of the transcription of pro-inflammatory genes (modified from [78]).

Accumulating evidence suggests that STAT1 phosphorylation on its serine residue, at position 727, is mandatory to the maximization of its transcriptional potency [88, 89]. This phosphorylation can be carried out by several serine kinases, such as MAPKs [87, 89].

It is important to bear in mind that the regulation of the biological actions of STAT proteins is also dependent on negative regulators [78]. For instance, suppressors of cytokine signalling (SOCs) proteins can block the recruitment of STATs by binding to IFNGR or JAKs' sites or even by targeting STATs for proteasomal degradation [76, 89]. Phosphatases, including SHP1 or SHP2, are another type of negative regulators of JAK/STAT pathway that can induce the dephosphorylation of JAKs and STATs, leading then to the inactivation of these proteins [75, 78, 88, 89]. More recently, another group of proteins called PIAS (protein inhibitors of activated STAT) have been investigated. PIAS constitute a family of proteins implicated in the inhibition of STAT-mediated gene activation through many mechanisms, namely inhibiting DNA binding and promoting the conjugation of small ubiquitin-like modifier (SUMO) proteins to STAT1 [76, 89, 90]. SUMOylation is a post-transcriptional modification that, in the case of STAT1, seems to negatively regulate STAT1-mediated genes responses, since it inhibits STAT1 Tyr701 phosphorylation, prevents STAT1 DNA binding and also promotes its dephosphorylation

[91-93].

Taken into account the above observations, it is possible to deduce that beyond NF-kB pathway, the modulation of JAK/STAT pathway, particularly of IFN-γ/STAT1 pathway, should be valued in the scope of IBD. In fact, beyond its contribution to the transcription of several pro-inflammatory and pro-oxidant genes, as already mentioned, this signalling cascade can also be involved in the acquisition of radiation resistance by patients, during radiotherapy treatment [94]. Given that IBD patients exhibit a high risk of developing colorectal cancer as compared to healthy population [95], the use of anti-inflammatory compounds with the potential to inhibit this pathway can be of redoubled interest for IBD patients. In the last years, *in vitro* and *in vivo* studies concerning the inhibition of JAK/STAT pathway, particularly of STAT1 and STAT3, have led to encouraging outcomes towards the amelioration of inflammation, namely in the colon [82, 83, 96-98]. Actually, a selective inhibitor of JAK1 is presently in development for the treatment of some inflammatory disorders, such as rheumatoid arthritis and CD [77]. This drug, Filgotinib (GPLG0634), is currently in phase II clinical trials for the treatment of CD, with favourable results already achieved in preclinical models for IBD [99].

2.3.3 Mitogen-Activated Protein Kinase (MAPK) pathway

Mitogen-activated protein kinases (MAPKs) are a group of evolutionary conserved serine/threonine kinases which coordinate several cellular activities, including cell proliferation, differentiation, survival and inflammation [100, 101]. The major subgroups of MAPKs are the extracellular signal-regulated kinases 1 and 2 (ERK 1/2), the c-Jun N-terminal kinases (JNKs) and the p38 kinases [102]. Although MAPKs are activated by different stimuli, ERK 1/2 are preferentially activated by growth factors and JNK and p38 MAPKs are specially responsive to environmental stress, such as osmotic shock, and to cytokines, such as TNF-α [64, 102]. The activation of each MAPK requires the prior operation of two sequential kinases: the MAPK kinase (MAPKK) and the MAPKK kinase (MAPKKK). MAPKKKs are serine/threonine kinases which phosphorylate (activate) MAPKKs, which in turn activate the MAPKs by dual phosphorylation on threonine and tyrosine residues [102]. MAPKs can be inactivated by phosphatases, such as dual-specificity phosphatases (DUSPs), threonine phosphatases and tyrosine phosphatases [103-105].

Given that both JNK and p38 MAPKs show to be highly activated in inflamed tissue of IBD patients, it is widely accepted that signal transduction pathways involving those enzymes are

implicated in this chronic intestinal inflammatory disease [104, 106, 107]. In fact, it is known their involvement in the modulation of the transcription activity of several pro-inflammatory transcription factors, such as NF-kB and STAT1, through phosphorylation [102, 108, 109]. In addition, particularly p38 MAPKs may also be involved in the post-transcriptional regulation of several important inflammatory genes, stabilizing, for instance, COX-2, iNOS and TNF-α mRNAs and promoting their translation [100, 103, 110]. Therefore, in the last years, there has been a considerable interest in the development of anti-JNK and anti-p38 therapies against intestinal inflammation [104-106]. Towards this goal, several studies have been carried out both in animal models and in humans [103, 104, 106, 111, 112]. However, the serious adverse effects reported in some clinical studies limited the application of some of these MAPK inhibitors [105]. More recently, some novel molecules have been tested in patients with IBD, such as Delmitide (RDP58) - an inhibitor of p38 and JNK, leading to significantly higher remission rates than that achieved with placebo and causing no serious adverse effects, which offers hope for the future use of these drugs in the IBD context [113].

2.3.4 Nuclear Factor-Erythroid-2-Related Factor 2 (Nrf2) pathway

Nuclear factor-erythroid-2-related factor 2 (Nrf2) pathway has emerged as a key regulator of cellular antioxidant and detoxifying defences against oxidative insult [114].

Under basal conditions, Nrf2 is sequestered in the cytoplasm, bound to the inhibitory protein Kelch-like ECH-associated protein 1 (Keap1), which has the ability to target Nrf2 for ubiquitination followed by proteasomal degradation [115]. The release of Nrf2 from Keap1 is neither fully understood nor consensual, but it is thought that dissociation of the transcription factor Nrf2 from its repressor occurs after alterations in the structure of Keap1 triggered by pro-oxidants or electrophiles (**Figure 10A**) or after phosphorylation of serine and/or threonine residues of Nrf2 by specific upstream kinases, namely MAPKs (**Figure 10B**) [114, 115]. Once free, Nrf2 is able to translocate to the nucleus to heterodimerize with other proteins, namely small Maf proteins, and to bind to antioxidant response elements (ARE), also called electrophile response elements (EpRE), in enhancer sites of responsive genes, promoting the expression of several important antioxidant and cytoprotective enzymes, such as hemoxygenase-1 (HO-1), glutamate cysteine ligase (GCL) and NAD(P)H:quinone oxidoreductase-1 (NQO1) [115, 116].

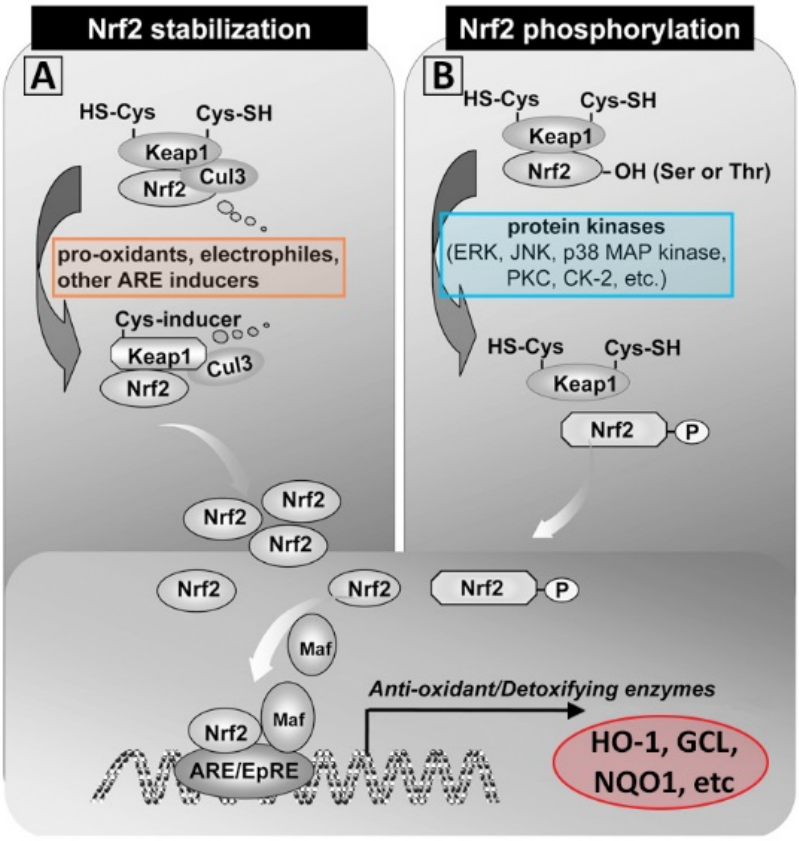


Figure 10 - Schematic drawing representing two possible mechanisms underlying the activation of Nrf2 pathway. A) Pro-oxidants or electrophiles induce the dissociation of Nrf2 from its repressor (Keap1) by producing specific alterations in the structure of Keap1. B) Upstream kinases, namely MAP Kinases, favour the dissociation of Nrf2 from Keap1 after phosphorylation of serine and/or threonine residues of Nrf2 (adapted from [115]).

Beyond the antioxidant protection afforded by Nrf2 activation, this transcription factor has demonstrated to be crucial in the suppression of the inflammatory process, among other mechanisms, by counteracting some pro-inflammatory cascades, as NF-kB pathway [116]. In line with this, accelerating research has shown that Nrf2-deficient mice have a heightened susceptibility to dextran sulfate sodium (DSS)-induced colitis [116, 117] and, for all of these reasons, Nrf2 pathway has gained increasing attention in the context of intestinal inflammation.

2.3.5 Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ) pathway

Peroxisome proliferator-activated receptors (PPARs) are members of a family of nuclear receptors, which are ligand-dependent transcription factors, involved in many biological processes, such as lipid metabolism, inflammation, and cell proliferation [118, 119]. Until now, three subtypes of PPARs were already found: PPAR-α, PPAR-δ/β and PPAR-γ [120, 121]. Since the recent findings that PPAR-γ subtype is highly expressed in colonic epithelial cells and in some intestinal immune cells, such as macrophages, being important for the maintenance of the intestinal homeostasis and the prevention of experimentally-induced colonic inflammatory injuries, this receptor has received considerable attention in the IBD context [118, 119]. Also, the latest relevant *in vitro* and *in vivo* studies, demonstrating that the anti-inflammatory effect of 5-aminosalicylic acid (non-steroid anti-inflammatory drug, commonly used in IBD patients) is dependent on the activation of PPAR-γ signalling cascade, have strengthened the importance of PPAR-γ as a target for IBD treatment [118, 122, 123].

In general terms, the induction of the transcriptional activity of PPAR-γ requires at first its binding to a specific agonist, then its heterodimerization with the nuclear retinoid X receptor (RXR) and further the PPAR/RXR heterodimer binding to sequence-specific regions in DNA, called peroxisome proliferator response elements (PPRE) [120]. The description made above corresponds to a conventional mechanism by which PPAR-γ positively regulates gene expression. However, through a mechanism (ligand-dependent) known as transrepression, PPAR-γ is able to negatively regulate gene expression. Transrepression is a complex mechanism, still under investigation, by which PPAR-γ antagonize the transcriptional activity of several transcription factors, such as NF-kB, STAT1 and AP-1, without interacting with PPRE (**Figure 11**) [120, 121].

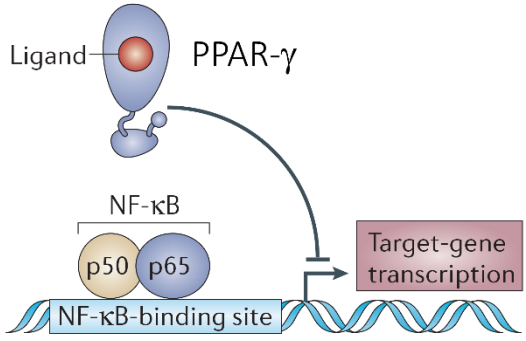


Figure 11 - Illustration for the mechanism of transrepression in which, in a ligand-dependent manner, PPAR-γ can antagonize NF-kB pathway (adapted from [121]).

This process has been invoked to explain the role of PPAR-γ against inflammation and also against oxidative stress [124, 125]. Transrepression has been explained by several mechanisms, including: a) direct protein-protein interaction between PPARs and other transcription factors, preventing the binding of the latter to sequence-specific regions in DNA; b) inhibition of the activity of upstream kinases, which modulate the activation of some transcription factors; c) competition for co-activator proteins that are required for the transcriptional activation of many transcription factors; d) prevention of the dissociation of co-repressors complexes from promoters, sustaining genes in a repressed state [120, 121, 126].

Therefore, it seems reasonable the demand to discover natural and synthetic ligands of PPAR-γ in order to limit IBD progression [118].

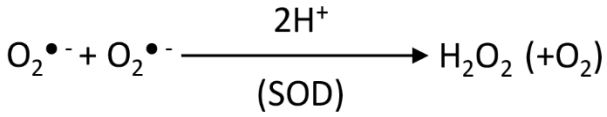
2.4 Oxidative stress and Inflammatory Bowel Disease

Reactive oxygen/nitrogen species (ROS/RNS) are highly reactive oxygen/nitrogen-derived small-molecules, constantly produced during physiological aerobic metabolism in mammalian cells, which, at low and moderate concentrations, may function as signalling molecules involved, for instance, in defence against invading pathogens [127, 128]. However, an excessive generation of ROS/RNS, not efficiently counteracted by an appropriate antioxidant defence system, leads to oxidative/nitrosative injury of the cells and tissues [127, 128]. Oxidative/nitrosative stress can be truly detrimental to cellular homeostasis, since it may sustain chronic inflammation and be responsible for lipid and protein modifications, DNA damage and apoptosis [128, 129].

2.4.1 Some relevant reactive oxygen/nitrogen species produced in the intestine

A - Reactive oxygen species (ROS)

Mitochondrial respiratory chain is the major source of ROS in mammalian cells [127]. During the oxidative phosphorylation, a small amount of molecular oxygen (O_2), instead of being fully reduced to water, generates the superoxide anion ($O_2^{\bullet -}$) resulting from the transfer of one sole electron to molecular oxygen [128]. In cells, superoxide anion is efficiently converted to hydrogen peroxide (H_2O_2) by superoxide dismutase [127, 130].



In physiological conditions, hydrogen peroxide is, then, converted to water by catalase or glutathione peroxidase [128]. Hydrogen peroxide that escapes from enzymatic degradation can be further reduced to the highly reactive hydroxyl radical ($HO^{\bullet}$), in a reaction catalysed by ferrous ion or by other transition metals, such as copper, chromium or cobalt (Fenton reaction) (**Figure 12**) [128]. The iron-catalysed Haber-Weiss reaction constitutes an important process through which hydroxyl radical is generated in biological systems, where the superoxide anion reduces the oxidized metal, released by the Fenton reaction, driving a reaction cycle [128].

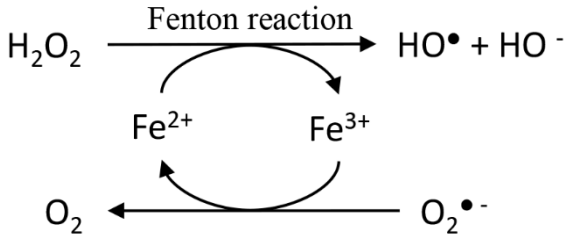


Figure 12 - Reaction cycle illustrating hydroxyl radical generation from superoxide anion and hydrogen peroxide (Haber-Weiss reaction).

It has been suggested that hydroxyl radical can be extremely harmful for the intestine, leading to the depolymerisation of mucin, peroxidation of lipids, oxidation of proteins and damage of mitochondrial RNA and DNA [128, 131].

Apart from mitochondria, NADPH oxidase (NOX) family is another crucial source of superoxide anion in cells [128]. NADPH oxidase is an enzymatic complex present in multiple cell-types, including in IECs and in phagocytes, such as neutrophils and macrophages, which catalyses electron transfer from NADPH to molecular oxygen, with the subsequently release of large amounts of superoxide anion. Phagocytes, such as macrophages, can degrade invading infectious agents by a process that involves the generation of superoxide anion, as well as other ROS, known as respiratory burst [127]. Some studies have suggested that intestinal macrophages from patients with CD have an augmented oxidative burst activity when compared with macrophages from healthy patients, and neutrophils from IBD patients are also able to generate higher amounts of superoxide anion than cells from healthy patients [131].

Furthermore, superoxide anion can also be generated by other enzymatic systems, such as xanthine oxidase and cyclooxygenase [127].

B - Reactive nitrogen species (RNS)

Nitric oxide (NO) is a free radical produced enzymatically by a family of enzymes called nitric oxide synthases (NOSs) [132]. All these enzymes catalyse the oxidation of L-arginine, leading to the production of L-citrulline and to the formation of nitric oxide (**Figure 13**) [127, 128, 132].

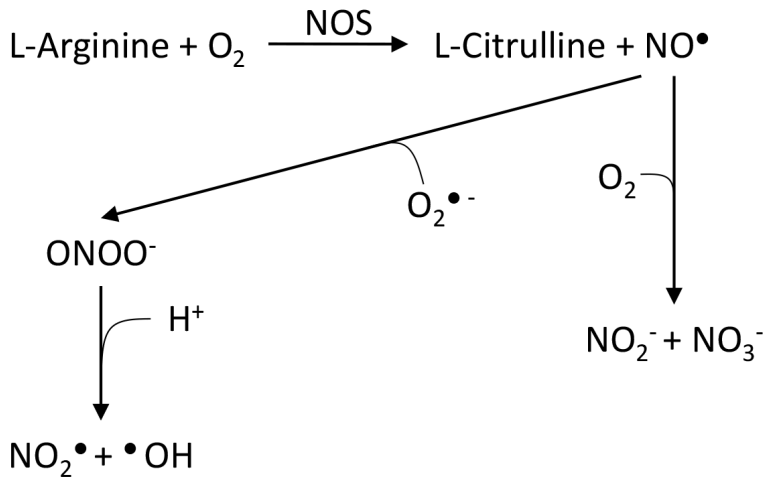


Figure 13 - Reactions of nitric oxide production and downstream reactive species generation.

Until now, three isoforms of NOSs were identified: the neuronal (nNOS) and the endothelial (eNOS), which are both constitutively expressed, and the inducible NOS (iNOS), whose expression is induced by some cytokines or bacterial products [127, 132].

As previously referred in points 2.3.1 and 2.3.2, several important transcription factors, namely NF-kB and STAT1, can promote, upon activation, the expression of iNOS in IECs. It is known that both the activation of NF-kB and the expression of iNOS are abnormally up-regulated in inflamed tissues of IBD patients [12, 131, 132]. Consistently, NO is continuously overproduced in these patients [131]. Noteworthy, moderate levels of NO seem to be crucial to maintain intestinal homeostasis, due to its ability to ensure a proper control of the blood flow and to regulate the epithelial permeability and the mucus production, among other mechanisms. In contrast, an excessive production of NO sustained for long periods of time, via up-regulation of iNOS, appears to contribute to the initiation and to the maintenance of intestinal inflammation, a condition observed in IBD patients and explained, for example, by its direct cytotoxicity or by the increase in the production of nitrosamines [127, 128, 132]. In

fact, NO is a free radical with a weak reactivity but that can react with superoxide anion to produce peroxynitrite anion ($ONOO^-$), which is a powerful cytotoxic oxidant capable of causing DNA fragmentation and lipid oxidation [127, 132]. Some studies show that the direct administration of peroxynitrite to colonic tissue is able to induce colitis in mice [127, 132]. Furthermore, as illustrated in **Figure 13**, peroxynitrite can become protonated and give rise to other free radicals, the nitric dioxide radical ($NO_2^{\bullet}$) and the hydroxyl radical ($OH^{\bullet}$). On the other hand, instead of reacting to superoxide anion, NO can interact with molecular oxygen and generates nitrite (NO_2^-) and nitrate (NO_3^-) ions (**Figure 13**), which, in turn, can give rise to highly reactive species, such as dinitrogen trioxide (N_2O_3) [127]. This molecule is a potent nitrosating agent able to N-nitrosate multiple molecules and to produce carcinogenic nitrosamines [132].

2.4.2 Antioxidant defence system

Due to the constant production of reactive species in the body, it is important that the antioxidant defence system is able to counterbalance this production in order to prevent the oxidative/nitrosative injury of the cells and tissues [131]. The antioxidant defence system can be organized in two main categories: the enzymatic antioxidants, including superoxide dismutases (SODs), catalase (CAT), glutathione peroxidase (GPX) and hemoxygenase (HO), and the non-enzymatic antioxidants, namely glutathione (GSH) and thioredoxin (TRX) [127, 130].

It has been suggested that patients with IBD have an inefficient antioxidant defence system, which make them more vulnerable to the effect of the overload of reactive species produced in the intestine, mainly during the active phases of this disease [12, 15, 131, 133]. For instance, pivotal studies conducted by Kruidenier *et al* [134, 135] pointed to an increase in the expression of mitochondrial manganese (Mn)-SOD, a SOD isoform, at the inflamed epithelium of patients with CD or UC, which was not accompanied by a parallel increase in CAT and/or GPX levels. As previously referred, SODs are crucial antioxidant enzymes that convert superoxide anion into hydrogen peroxide, which, in turn, is further neutralized to water by CAT or GPX [127]. In physiological conditions, it is of paramount importance that the formation of hydrogen peroxide does not exceed its removal and, therefore, the activity of those antioxidant enzymes needs to be strictly coordinated. In this context, the reported increase in the expression of Mn-SOD not accompanied by a subsequent increase in the expression of CAT and/or GPX,

suggesting a deficient removal of hydrogen peroxide and a possible increase in hydroxyl radical levels (through Fenton reaction), should contribute to the oxidative damage observed in the intestine of IBD patients [134, 135].

On the other hand, it has also been suggested that levels of GSH are reduced in the intestine of IBD patients, which can have a great impact on both enzymatic and non-enzymatic antioxidant defences, rendering hence intestinal cells more susceptible to oxidative injury [12, 131].

2.5 Chronic inflammation, oxidative stress and Inflammatory Bowel Disease – gathering loose ends

Although the etiology of IBD is not fully understood, it is known that an exacerbated and uncontrolled inflammatory response to bacteria that normally inhabit in the gastrointestinal tract is triggered in a genetically susceptible individual [12, 131]. It is also known that the overproduction of pro-inflammatory mediators, namely cytokines and chemokines, allied to the excessive generation of ROS/RNS by IECs and by immune cells, is not efficiently counterbalanced by the anti-inflammatory and antioxidant mechanisms that normally ensure the recovery of intestinal homeostasis in physiological situations, which explains the perpetuation of intestinal inflammation and oxidative stress, verified in IBD patients [12, 15, 131, 136]. In line with this, growing evidence suggests that inflammation and oxidative stress play a crucial role in the etiology and in the progression of IBD (**Figure 14**) [12, 15, 127, 128, 133].

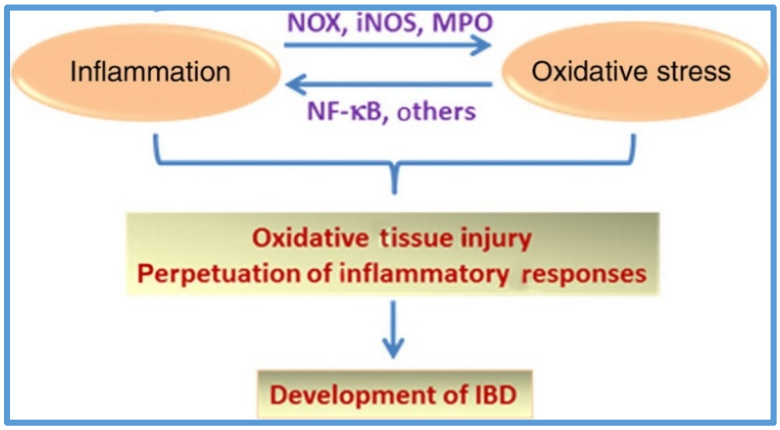


Figure 14 - Picture representing the intricate relationship between inflammation and oxidative stress and their contribution to the development of IBD (adapted from [15]).

At the cellular level, there are several examples that illustrate the intertwined relationship between chronic inflammation and oxidative stress, in a mechanistic perspective. Although this matter is still under intense investigation, it is thought that oxidative stress can keep active the inflammatory response by inducing an important pro-inflammatory redox-sensitive transcription factor, the NF-kB [137]. On the other hand, NF-kB, itself, can lead to the generation of further amounts of ROS/RNS, contributing to the maintenance of this vicious cycle, that, in turn, eventually results in serious damage to the intestinal barrier [12].

2.6 Conventional pharmacological approaches: a still unsolved issue

Until the present, IBD does not have cure and the pharmacological treatment is mainly used to prevent relapses, to induce or maintain the remission periods, to treat intestinal or extra-intestinal complications and to improve the quality of life of the patients [12, 138, 139].

In fact, as illustrated in the therapeutic pyramid below (**Figure 15**), the drugs conventionally prescribed for the treatment of IBD can be divided in several groups, including: aminosalicylates, corticosteroids, immunomodulators and biologic agents [140].

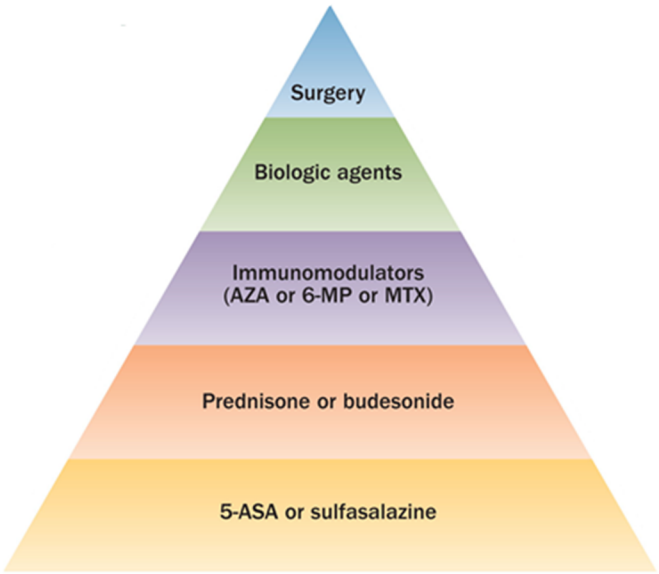


Figure 15 - Therapeutic pyramid of the drugs conventionally prescribed for the treatment of IBD patients. 5-ASA, 5-aminosalicylic acid; AZA, azathioprine; 6-MP, 6-mercaptopurine; MTX, methotrexate (adapted from [141]).

2.6.1 Aminosalicylates

Sulfasalazine was the first aminosalicylate used in IBD and it is formed by the combination of 5-aminosalicylic acid (the active moiety) to sulfapyridine, linked by an azo bond, functioning as a prodrug [138]. The sulfapyridine group is used as a carrier of the 5-aminosalicylic acid (5-ASA), delivering it in the colon, after cleavage of the azo bond by the colonic bacterial azoreductase, thus avoiding an early absorption of the active compound in the upper regions of gastrointestinal tract [138, 142]. However, the existence of the sulfapyridine group makes sulfasalazine poorly tolerated by many patients and so, it was excluded from the novel formulations of 5-ASA [138, 143]. In the last years, there has been a great effort in developing increasingly sophisticated oral-5-ASA delivery systems, which have been designed in order to delay and/or sustain the release of 5-ASA in specific inflamed regions of the gastrointestinal tract [144]. Furthermore, there are also rectal formulations of 5-ASA, which can also be important to manage intestinal inflammation, often combined with oral forms [95, 143].

5-ASA is envisaged as a “topical agent”, acting locally in the gastrointestinal tract [95, 142]. Accordingly a recent review by Williams *et al* [95], the clinical effectiveness of 5-ASA depends mostly on the mucosal concentration achieved in the gastrointestinal tract rather than on the systemic absorption and on the subsequent redistribution of the drug to the gastrointestinal tract.

To date, the action mechanisms underlying the clinical efficacy of 5-ASA remain not fully understood. Growing data suggest that 5-ASA is able to inhibit the production of pro-inflammatory mediators, such as NO and PGE_2, and the expression of pro-inflammatory enzymes, including iNOS and COX-2. These effects are generally correlated to the ability of 5-ASA to inhibit NF-kB signalling pathway [123]. On the other hand, since it was found that 5-ASA is an agonist of PPAR-γ, attention has been paid to the possible involvement of this receptor in the anti-inflammatory activity of 5-ASA [123, 139]. The antioxidant activity of 5-ASA in *in vitro* studies as well as clinical evidences supporting its radical scavenger ability have also been reported, suggesting that this action mechanism can contribute to attenuate intestinal inflammation [145, 146].

In our days, 5-ASA is considered the first-line therapy for the treatment of mild to moderate UC (which accounts for 90% of patients with UC, at first presentation), and for the maintenance of the remission periods in these patients [142]. In the case of CD patients, the beneficial effects of 5-ASA can be limited. However, although the use of 5-ASA in CD patients is not consensual, it is still widely used in clinical practice [8, 95, 138, 140].

Concerning potential side effects of 5-ASA, it is known that this drug is often well-tolerated but it is not free of adverse effects, diarrhoea, nauseas, dyspepsia, skin rashes and headache being the most common [138, 143]. Furthermore, nephrotoxicity may occur sometimes and, for this reason, the renal function of the patients receiving this drug should be closely monitored [95, 138].

2.6.2 Other pharmacological options for Inflammatory Bowel Disease treatment

Beyond aminosalicylates, there are other alternative drugs used in the treatment of IBD. As depicted in **Figure 15**, the treatment of IBD involves, normally, a "step-up approach", which means that the most aggressive therapies are only selected when the softer therapies fail or are not sufficiently effective for the degree of severity of the disease [138, 147]. Therefore, the first pharmacological option for IBD patients is usually aminosalicylates, and for this reason they are positioned at the base of the pyramid represented in **Figure 15** [8, 147]. However, if patients do not respond to aminosalicylates, the next therapeutic option often consists of using corticosteroids [8, 147]. Corticosteroids are a group of potent anti-inflammatory compounds used mainly for moderate to severe acute flares in IBD patients [148]. Steroids can be administered orally, rectally or intravenously [148]. Due to their typical adverse effects, their use must be avoided for long-periods and so they are not recommended to maintain the remission periods in patients with IBD [138]. The usual adverse effects of steroids include osteoporosis, Cushing syndrome and susceptibility to infection [148]. When corticosteroids fail or are not adequate, the next approach is usually the administration of immunomodulators, such as azathioprine and 6-marcaptopurine [147]. These drugs can be particularly useful in specific cases, such as to maintain the remission in IBD patients not responding to aminosalicylates or intolerant to them [147]. However, it is estimated that up to 20% of patients has to stop this therapy because of the harmful side effects, namely severe gastrointestinal symptoms, hepatotoxicity and pancreatitis [140].

Infliximab and adalimumab are some of the anti-TNF-α monoclonal antibodies, which belong to the group of biologic agents used, nowadays, as a powerful pharmacological option in IBD, particularly in patients with moderate to severe CD, who are refractory, have contraindications or poor tolerance to all other pharmaceutical drugs and in patients with severe UC not responding to any other drugs [140]. The most serious and common adverse effects of these therapeutics are opportunistic infections, malignancies and autoimmunity [140].

Despite all the therapeutic weapons currently available for the management of IBD, the

results achieved are clearly unsatisfactory, since many patients often become refractory or intolerant to the therapy and both long-term side effects and the necessity of surgery become almost inevitable [12].

In this way, it is still urgent to find preventive strategies and better targeted therapies effective in limiting IBD progression.

3. Polyphenols and their benefits in health promotion and disease prevention

Polyphenols are a wide group of plant secondary metabolites, which contribute to plant pigmentation, growth, pollination and resistance against environmental stress, pathogens and predators [68, 149-151].

Concerning their chemical structure, polyphenols are generically characterized by the presence of one or more hydroxyl groups linked to a benzene ring and can be classified as flavonoids and nonflavonoids, as illustrated in **Figure 16** [149, 150, 152]. Flavonoids are a group of polyphenolic compounds with a common structure (two benzene rings linked through a linear three-carbon chain, forming an oxygenated heterocycle, C_6-C_3-C_6) and can be subdivided into several subgroups, including flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins [149, 150]. The nonflavonoids group includes structurally different compounds, such as phenolic acids (C_6-C_1), lignans (C_6-C_3-C_3-C_6) and stilbenes (C_6-C_2-C_6) [153].

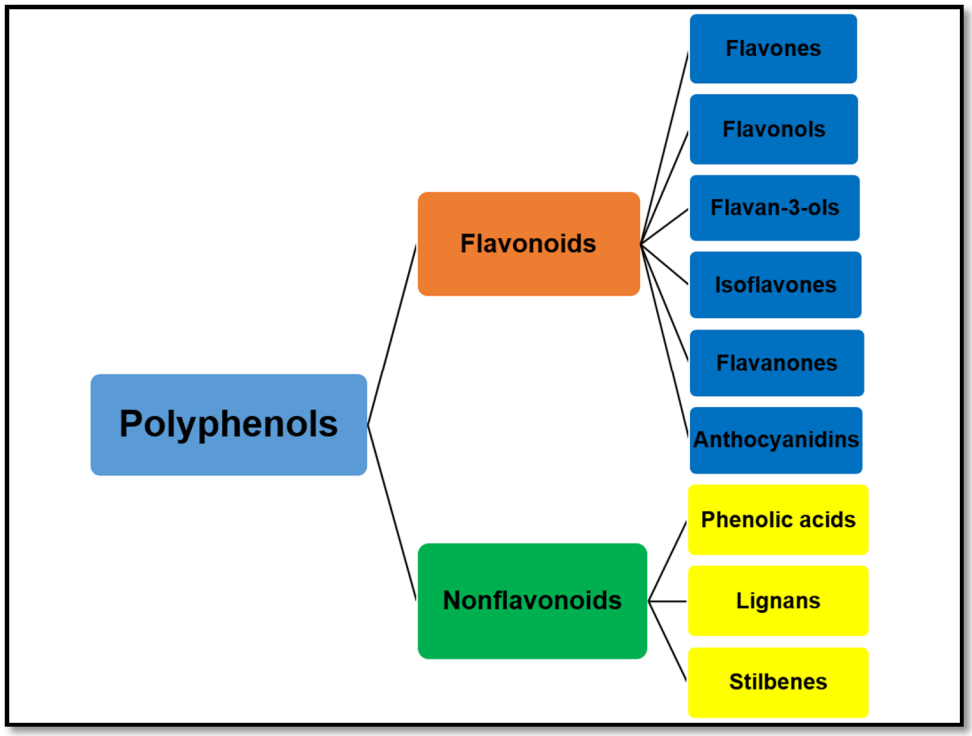


Figure 16 - Schematic picture representing the classification of polyphenols in two groups, flavonoids and nonflavonoids, and their respective subgroups.

In nature, polyphenols are commonly linked to sugars, such as glucose or rhamnose, through one or more hydroxyl groups, being generally referred to as glycosylated compounds [151, 154]. When not attached to sugars, polyphenols are referred to as being in their aglycone form [151].

Until now, more than 8000 polyphenols have been identified [68, 152]. It is known that these compounds are highly present in human diet, through the consumption of fruits, vegetables, cereals and beverages, such as tea, coffee and wine [152, 155]. Red-wine, for instance, is one of the most rich sources of polyphenols, being very appreciated in Western Societies and taking part of the Mediterranean diet, which was recently recognized by The United Nations Educational, Scientific and Cultural Organization (UNESCO) as an intangible cultural heritage of humanity [130, 156, 157].

In the last decades, a flourishing research field has been focused on the study of the positive effects of several dietary polyphenols in the prevention and treatment of multiple chronic inflammatory disorders, such as cardiovascular disease, type II diabetes, obesity, arthritis and IBD [130, 149, 154, 158]. Although the beneficial effects of polyphenolic compounds had been initially attributed almost exclusively to their antioxidant capacity, increasing evidence has currently proven that such beneficial effects are strongly supported by the ability of dietary polyphenols to interfere with essential signalling pathways and with gene regulation [149, 154, 158]. However, the interactions established between polyphenols and cellular targets and the precise signalling pathways underlying polyphenol effects are far from being fully elucidated.

It is known that the structural characteristics of polyphenols are crucial to determine their ability to be adsorbed and to penetrate into the lipid bilayer of the cells. Furthermore, even if they are not internalized, it is also known that polyphenols can still modulate the activity of membrane-associated enzymes and to regulate the transduction of signals, thanks to modifications of the plasma membrane structure and physical properties, namely fluidity and electrical properties, among other mechanisms [149]. Actually, very recent studies have shown that some polyphenols, such as resveratrol, cyanidin-3-glucoside and epigallocatechingallate (ECGG), can interact with specialized domains of cell membranes, known as lipid rafts, which are characterized by possessing a specific lipid composition, enriched in sphingomyelin and cholesterol, and by containing proteins related to the membrane signalling and trafficking [149, 159, 160]. Thus, some studies suggest that the interaction of resveratrol with lipid rafts is implicated in the activation of downstream signalling pathways, leading to the cell death of several cancer cells [160], whereas the disruption of lipid rafts provoked by

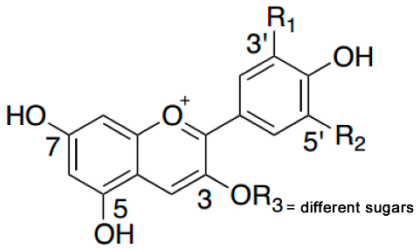
cyanidin-3-glucoside appears to be crucial for the suppression of inflammation in macrophages [159].

Apart from the interaction of polyphenols with membranes, polyphenols can also interact specifically with enzymes, receptors and transcription factors, depending on their chemical features, thus addressing multiple biological targets, which could be potentially advantageous for disease prevention or treatment [149]. This is illustrated by some polyphenols, such as flavanols, which can apparently inhibit NF-kB activation in certain *in vitro* models, by direct interaction with NF-kB proteins, preventing their binding to DNA kB sites [149]. Given the great involvement of NF-kB pathway in the development of IBD, the inhibition of this pathway by dietary polyphenols is considered a promising strategy for the prevention of IBD progression.

Although the low oral bioavailability of the majority of polyphenols can constitute a great handicap for its use in the context of many pathologies [161], in the particular case of intestinal diseases, including IBD, the poor oral bioavailability achieved is not, paradoxically, a true problem, since several studies claim that the intestine is the organ where dietary polyphenols achieve the highest concentrations in the human body, approximately up to several hundred µM [68, 156, 161].

Given the great diversity of dietary polyphenols, the present Thesis is focused on two important subgroups of polyphenolic compounds typically present in the Mediterranean diet: the anthocyanidins, which belong to the large group of flavonoids, and the stilbenes, which belong to the group of nonflavonoids. Although there are approximately seventeen anthocyanidins in the nature, cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin are the most relevant for the human diet, and among them, cyanidin is the anthocyanidin most commonly found in foods [155, 162]. In nature, these compounds normally occur in their glycosylated form, termed anthocyanin, rather than in their aglycone form, referred as anthocyanidin, since they are generally associated with sugars, such as glucose, galactose, rhamnose, arabinose and xylose, forming mono-, di- or trisaccharides [154, 162-164].

Anthocyanins (*anthos*, in Greek, means flower and *kyanos* means blue) are water-soluble plant pigments responsible for the blue, purple and red colours of many fruits, such as blueberries, blackberries, raspberries, strawberries and red grapes (**Figure 17**) [162, 163, 165]. Anthocyanins are therefore present in fruit-derived beverages, such as red-wine. It has been demonstrated that wine contains about 200-350 mg anthocyanins per liter [155].



Anthocyanin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 17 - Chemical structure of anthocyanins.

Chemically, anthocyanins are glycosylated, polyhydroxy or polymethoxy derivative forms of 2-phenylbenzopyrylium, with two benzoyl rings and a heterocyclic ring in the middle, being considered really unique flavonoids, since they can assume different chemical structures in aqueous solution, according to the pH and temperature [165]. It is known that the red flavylium cation is the most abundant form at very acidic pH (pH < 2) and, with the increase of pH, there is a rapid structural re-arrangement of its structure thanks to the loss of a proton, leading to the appearance of the blue quinonoidal form. However, the flavylium cation also undergoes a slow hydration step, yielding the uncoloured hemiketal form, which, in turn, further tautomerises to a chalcone (*cis* and *trans* forms) [165]. These re-arrangements of the anthocyanins' structure should not be undervalued, since during the passage in the human gastrointestinal tract, the pH undergoes large variations and consequently the chemical forms will possibly vary as well as their bioactive properties [165].

In what concerns stilbenes, the commonest compound of this group is resveratrol. Resveratrol is predominantly found in the skin of red grapes, in some berries and peanuts [154]. Moreover, red-wine, rather than white-wine, contains significant amounts of this compound. Chemically, resveratrol is composed of two aromatic rings joined by an ethylene bridge (**Figure 18**) [166]. Although resveratrol can exist in either *trans* or *cis* form, the preferred steric form of this compound is the *trans* form [158]. Resveratrol can also exist in aglycone or in glycosylated form [149].

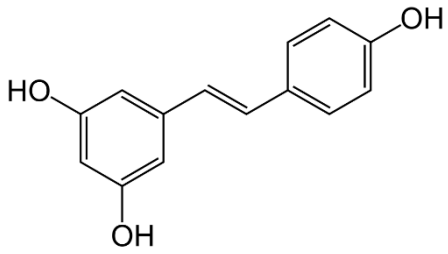


Figure 18 - Chemical structure of trans-resveratrol (aglycone form).

In the last decades, either anthocyanins or resveratrol have been the focus of intensive research mainly in the context of the prevention of oxidative stress-associated diseases, such as atherosclerosis and diabetes, not only due to their antioxidant capacity but also due to their anti-inflammatory activity [167-169]. In fact, recent studies underscore their capacity as inhibitors of inflammation-related transcriptions factors, particularly of NF-kB, and of inflammatory markers, namely iNOS, COX-2 and cytokines [162, 168, 170, 171]. Therefore, interest has been raised in using polyphenols as promising nutraceuticals, capable of giving complementary benefits for the prevention and/or treatment of chronic inflammatory diseases, as the case of IBD [154].

4. Objectives

Given that, until now, Inflammatory Bowel Disease (IBD) does not have cure and the currently available pharmacological options are long-life treatments, which often bring severe adverse effects, it remains quite urgent to find safer and better targeted strategies efficient to limit IBD progression. On the other hand, dietary polyphenols are a group of compounds of natural origin with great abundance in fruits, vegetables, green tea and red-wine, which have been the focus of intensive research in the context of the prevention of several chronic inflammatory diseases, but whose cellular mechanisms underlying their claimed beneficial effects are not fully clarified. Therefore, the hypothesis from which the work presented in this Thesis has emerged is that natural compounds widespread in foods typically present in a Mediterranean diet are able to limit IBD progression, at the concentration range predictably reached at the intestinal level, with an efficiency similar or superior to a well-established therapeutic intervention, commonly prescribed for IBD patients. Therefore, the main goal of this work is to assay two structurally different polyphenolic compounds, one flavonoid, the anthocyanin cyanidin-3-glucoside (C3G), and one nonflavonoid, the stilbene resveratrol (Resv), both present in the Mediterranean diet, to evaluate their effects against inflammation and oxidative stress, which typically characterize IBD, as compared to the well-known anti-inflammatory drug, 5-aminosalicylic acid (5-ASA), in a human intestinal cell line (HT-29) as a model.

In order to test our hypothesis and to achieve our main goal, we propose to carry out several tasks with the following specific objectives:

1. To assess the ability of cyanidin-3-glucoside to suppress the inflammatory response induced by a combination of inflammatory cytokines, in comparison with the drug 5-aminosalicylic acid, in HT-29 cells. In this context, the ability of C3G to counteract the expression of crucial pro-inflammatory mediators or the activation of transcription factors with a recognized importance in the development of IBD, such as NF-kB and JAK/STAT pathways, was evaluated (Chapter 2).

2. To extend the previous research to a structurally different polyphenol - the well-known resveratrol - in order to elucidate, under a mechanistic perspective, its capacity of inhibiting the inflammatory process, at the same experimental conditions used for C3G, in comparison with 5-aminosalicylic acid. The cell signalling cascades underlying Resv anti-inflammatory effect, including JNK and p38 MAPK pathways, beyond those of NF-kB and JAK/STAT, were

ascertained (Chapter 3).

3. To elucidate the implication of Nrf2 and PPAR-γ in the protection afforded by cyanidin-3-glucoside and resveratrol against inflammation and oxidative stress, using the same *in vitro* model and making comparison with the drug 5-aminosalicylic acid (Chapter 4).

5. References

1. Peterson, L.W. & Artis, D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol* **14**, 141-53 (2014).
2. Maynard, C.L., Elson, C.O., Hatton, R.D. & Weaver, C.T. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* **489**, 231-41 (2012).
3. Hooper, L.V. & Macpherson, A.J. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* **10**, 159-69 (2010).
4. Brown, E.M., Sadarangani, M. & Finlay, B.B. The role of the immune system in governing host-microbe interactions in the intestine. *Nat Immunol* **14**, 660-7 (2013).
5. Goto, Y. & Ivanov, II. Intestinal epithelial cells as mediators of the commensal-host immune crosstalk. *Immunol Cell Biol* **91**, 204-14 (2013).
6. Artis, D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev Immunol* **8**, 411-20 (2008).
7. Maloy, K.J. & Powrie, F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* **474**, 298-306 (2011).
8. Baumgart, D.C. & Sandborn, W.J. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* **369**, 1641-57 (2007).
9. Lichtenstein, G.R., Hanauer, S.B., Sandborn, W.J. & Practice Parameters Committee of American College of, G. Management of Crohn's disease in adults. *Am J Gastroenterol* **104**, 465-83 (2009).
10. Kornbluth, A., Sachar, D.B. & Practice Parameters Committee of the American College of, G. Ulcerative colitis practice guidelines in adults: American College Of Gastroenterology, Practice Parameters Committee. *Am J Gastroenterol* **105**, 501-23 (2010).
11. Baumgart, D.C. & Carding, S.R. Inflammatory bowel disease: cause and immunobiology. *Lancet* **369**, 1627-40 (2007).
12. Biasi, F., Leonarduzzi, G., Oteiza, P.I. & Poli, G. Inflammatory bowel disease: mechanisms, redox considerations, and therapeutic targets. *Antioxid Redox Signal* **19**, 1711-47 (2013).
13. Bernstein, C.N. et al. World Gastroenterology Organization Practice Guidelines for the diagnosis and management of IBD in 2010. *Inflamm Bowel Dis* **16**, 112-24 (2010).
14. Sartor, R.B. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative

colitis. *Nat Clin Pract Gastroenterol Hepatol* **3**, 390-407 (2006).

15. Zhu, H. & Li, Y.R. Oxidative stress and redox signalling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. *Exp Biol Med* **237**, 474-80 (2012).
16. Neurath, M.F. New targets for mucosal healing and therapy in inflammatory bowel diseases. *Mucosal Immunol* **7**, 6-19 (2014).
17. Bouma, G. & Strober, W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* **3**, 521-33 (2003).
18. Mowat, C. et al. Guidelines for the management of inflammatory bowel disease in adults. *Gut* **60**, 571-607 (2011).
19. Mowat, A.M. & Agace, W.W. Regional specialization within the intestinal immune system. *Nat Rev Immunol* **14**, 667-85 (2014).
20. Cader, M.Z. & Kaser, A. Recent advances in inflammatory bowel disease: mucosal immune cells in intestinal inflammation. *Gut* **62**, 1653-64 (2013).
21. Turner, J.R. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* **9**, 799-809 (2009).
22. Coskun, M. Intestinal epithelium in inflammatory bowel disease. *Front Med* **1**, 24-29 (2014).
23. Laukoetter, M.G., Nava, P. & Nusrat, A. Role of the intestinal barrier in inflammatory bowel disease. *World J Gastroenterol* **14**, 401-7 (2008).
24. Kim, Y.S. & Ho, S.B. Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Curr Gastroenterol Rep* **12**, 319-30 (2010).
25. Moehle, C. et al. Aberrant intestinal expression and allelic variants of mucin genes associated with inflammatory bowel disease. *J Mol Med (Berl)* **84**, 1055-66 (2006).
26. Sheng, Y.H., Hasnain, S.Z., Florin, T.H. & McGuckin, M.A. Mucins in inflammatory bowel diseases and colorectal cancer. *J Gastroenterol Hepatol* **27**, 28-38 (2012).
27. Gallo, R.L. & Hooper, L.V. Epithelial antimicrobial defence of the skin and intestine. *Nat Rev Immunol* **12**, 503-16 (2012).
28. Fellermann, K. et al. A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. *Am J Hum Genet* **79**, 439-48 (2006).
29. Johansen, F.E. & Kaetzel, C.S. Regulation of the polymeric immunoglobulin receptor and IgA transport: new advances in environmental factors that stimulate pIgR

expression and its role in mucosal immunity. *Mucosal Immunol* **4**, 598-602 (2011).

30. Macpherson, A., Khoo, U.Y., Forgacs, I., Philpott-Howard, J. & Bjarnason, I. Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. *Gut* **38**, 365-75 (1996).
31. Matricon, J., Barnich, N. & Ardid, D. Immunopathogenesis of inflammatory bowel disease. *Self Nonself* **1**, 299-309 (2010).
32. Cario, E. Toll-like receptors in inflammatory bowel diseases: a decade later. *Inflamm Bowel Dis* **16**, 1583-97 (2010).
33. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11**, 373-84 (2010).
34. Mogensen, T.H. Pathogen recognition and inflammatory signalling in innate immune defenses. *Clin Microbiol Rev* **22**, 240-73 (2009).
35. Creagh, E.M. & O'Neill, L.A. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol* **27**, 352-7 (2006).
36. Abreu, M.T., Fukata, M. & Arditi, M. TLR signalling in the gut in health and disease. *J Immunol* **174**, 4453-60 (2005).
37. Abreu, M.T. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol* **10**, 131-44 (2010).
38. Lavelle, E.C., Murphy, C., O'Neill, L.A. & Creagh, E.M. The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. *Mucosal Immunol* **3**, 17-28 (2010).
39. Kamada, N., Seo, S.U., Chen, G.Y. & Nunez, G. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol* **13**, 321-35 (2013).
40. Baumgart, D.C. & Sandborn, W.J. Crohn's disease. *Lancet* **380**, 1590-605 (2012).
41. Salim, S.Y. & Soderholm, J.D. Importance of disrupted intestinal barrier in inflammatory bowel diseases. *Inflamm Bowel Dis* **17**, 362-81 (2011).
42. Khor, B., Gardet, A. & Xavier, R.J. Genetics and pathogenesis of inflammatory bowel disease. *Nature* **474**, 307-17 (2011).
43. Fukata, M. & Arditi, M. The role of pattern recognition receptors in intestinal inflammation. *Mucosal Immunol* **6**, 451-63 (2013).
44. Magalhaes, J.G., Tattoli, I. & Girardin, S.E. The intestinal epithelial barrier: how to distinguish between the microbial flora and pathogens. *Semin Immunol* **19**, 106-15 (2007).
45. Mowat, A.M. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat*

Rev Immunol **3**, 331-41 (2003).

46. Cheroutre, H. & Madakamutil, L. Acquired and natural memory T cells join forces at the mucosal front line. *Nat Rev Immunol* **4**, 290-300 (2004).
47. Jump, R.L. & Levine, A.D. Mechanisms of natural tolerance in the intestine: implications for inflammatory bowel disease. *Inflamm Bowel Dis* **10**, 462-78 (2004).
48. Kambayashi, T. & Laufer, T.M. Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? *Nat Rev Immunol* **14**, 719-30 (2014).
49. Cerovic, V., Bain, C.C., Mowat, A.M. & Milling, S.W. Intestinal macrophages and dendritic cells: what's the difference? *Trends Immunol* **35**, 270-7 (2014).
50. Rescigno, M. & Di Sabatino, A. Dendritic cells in intestinal homeostasis and disease. *J Clin Invest* **119**, 2441-50 (2009).
51. Farache, J., Zigmond, E., Shakhar, G. & Jung, S. Contributions of dendritic cells and macrophages to intestinal homeostasis and immune defense. *Immunol Cell Biol* **91**, 232-9 (2013).
52. Abraham, C. & Medzhitov, R. Interactions between the host innate immune system and microbes in inflammatory bowel disease. *Gastroenterology* **140**, 1729-37 (2011).
53. Arseneau, K.O., Tamagawa, H., Pizarro, T.T. & Cominelli, F. Innate and adaptive immune responses related to IBD pathogenesis. *Curr Gastroenterol Rep* **9**, 508-12 (2007).
54. Di Sabatino, A., Biancheri, P., Rovedatti, L., MacDonald, T.T. & Corazza, G.R. New pathogenic paradigms in inflammatory bowel disease. *Inflamm Bowel Dis* **18**, 368-71 (2012).
55. Spellberg, B. & Edwards, J.E., Jr. Type 1/Type 2 immunity in infectious diseases. *Clin Infect Dis* **32**, 76-102 (2001).
56. Xavier, R.J. & Podolsky, D.K. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **448**, 427-34 (2007).
57. Neurath, M.F. Cytokines in inflammatory bowel disease. *Nat Rev Immunol* **14**, 329-42 (2014).
58. Ordas, I., Eckmann, L., Talamini, M., Baumgart, D.C. & Sandborn, W.J. Ulcerative colitis. *Lancet* **380**, 1606-19 (2012).
59. Wittkopf, N., Neurath, M.F. & Becker, C. Immune-epithelial crosstalk at the intestinal surface. *J Gastroenterol* **49**, 375-87 (2014).
60. Wells, J.M., Rossi, O., Meijerink, M. & van Baarlen, P. Epithelial crosstalk at the

microbiota-mucosal interface. *Proc Natl Acad Sci* **108 Suppl 1**, 4607-14 (2011).

61. Uwe, S. Anti-inflammatory interventions of NF-kappaB signalling: potential applications and risks. *Biochem Pharmacol* **75**, 1567-79 (2008).
62. Oeckinghaus, A., Hayden, M.S. & Ghosh, S. Crosstalk in NF-kappaB signalling pathways. *Nat Immunol* **12**, 695-708 (2011).
63. Pasparakis, M. Regulation of tissue homeostasis by NF-kappaB signalling: implications for inflammatory diseases. *Nat Rev Immunol* **9**, 778-88 (2009).
64. Perkins, N.D. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* **8**, 49-62 (2007).
65. Wullaert, A., Bonnet, M.C. & Pasparakis, M. NF-kappaB in the regulation of epithelial homeostasis and inflammation. *Cell Res* **21**, 146-58 (2011).
66. Li, Q. & Verma, I.M. NF-kappaB regulation in the immune system. *Nat Rev Immunol* **2**, 725-34 (2002).
67. Lawrence, T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol* **1**, a001651 (2009).
68. Romier, B., Schneider, Y.J., Larondelle, Y. & During, A. Dietary polyphenols can modulate the intestinal inflammatory response. *Nutr Rev* **67**, 363-78 (2009).
69. Gloire, G., Legrand-Poels, S. & Piette, J. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochem Pharmacol* **72**, 1493-505 (2006).
70. Hoffmann, A. & Baltimore, D. Circuitry of nuclear factor kappaB signalling. *Immunol Rev* **210**, 171-86 (2006).
71. Pasparakis, M. Role of NF-kappaB in epithelial biology. *Immunol Rev* **246**, 346-58 (2012).
72. Rogler, G. et al. Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology* **115**, 357-69 (1998).
73. Atreya, I., Atreya, R. & Neurath, M.F. NF-kappa B in inflammatory bowel disease. *J Intern Med* **263**, 591-596 (2008).
74. Pasparakis, M. IKK/NF-kappaB signalling in intestinal epithelial cells controls immune homeostasis in the gut. *Mucosal Immunol* **1 Suppl 1**, S54-7 (2008).
75. Rane, S.G. & Reddy, E.P. Janus kinases: components of multiple signalling pathways. *Oncogene* **19**, 5662-79 (2000).
76. Santos, C.I. & Costa-Pereira, A.P. Signal transducers and activators of transcription-from cytokine signalling to cancer biology. *Biochim Biophys Acta* **1816**,

38-49 (2011).

77. O'Shea, J.J. et al. The JAK-STAT pathway: impact on human disease and therapeutic intervention. *Annu Rev Med* **66**, 311-28 (2015).
78. Levy, D.E. & Darnell, J.E., Jr. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* **3**, 651-62 (2002).
79. Ito, R. et al. Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice. *Clin Exp Immunol* **146**, 330-8 (2006).
80. Schroder, K., Hertzog, P.J., Ravasi, T. & Hume, D.A. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**, 163-89 (2004).
81. Coskun, M., Salem, M., Pedersen, J. & Nielsen, O.H. Involvement of JAK/STAT signalling in the pathogenesis of inflammatory bowel disease. *Pharmacol Res* **76**, 1-8 (2013).
82. Rauch, I., Muller, M. & Decker, T. The regulation of inflammation by interferons and their STATs. *JAKSTAT* **2**, e23820 (2013).
83. Midura-Kiela, M.T. et al. Curcumin inhibits interferon-gamma signalling in colonic epithelial cells. *Am J Physiol Gastrointest Liver Physiol* **302**, G85-96 (2012).
84. Singh, U.P., Venkataraman, C., Singh, R. & Lillard, J.W., Jr. CXCR3 axis: role in inflammatory bowel disease and its therapeutic implication. *Endocr Metab Immune Disord Drug Targets* **7**, 111-23 (2007).
85. Kuwano, Y. et al. Interferon-gamma activates transcription of NADPH oxidase 1 gene and upregulates production of superoxide anion by human large intestinal epithelial cells. *Am J Physiol Cell Physiol* **290**, C433-43 (2006).
86. Stempelj, M., Kedinger, M., Augenlicht, L. & Klampfer, L. Essential role of the JAK/STAT1 signalling pathway in the expression of inducible nitric-oxide synthase in intestinal epithelial cells and its regulation by butyrate. *J Biol Chem* **282**, 9797-804 (2007).
87. de Prati, A.C. et al. STAT1 as a new molecular target of anti-inflammatory treatment. *Curr Med Chem* **12**, 1819-28 (2005).
88. Ramana, C.V., Chatterjee-Kishore, M., Nguyen, H. & Stark, G.R. Complex roles of Stat1 in regulating gene expression. *Oncogene* **19**, 2619-27 (2000).
89. Shuai, K. & Liu, B. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol* **3**, 900-11 (2003).
90. Klampfer, L. Signal transducers and activators of transcription (STATs): Novel targets

of chemopreventive and chemotherapeutic drugs. *Curr Cancer Drug Targets* **6**, 107-21 (2006).

91. Gronholm, J. et al. Structure-function analysis indicates that sumoylation modulates DNA-binding activity of STAT1. *BMC Biochem* **13**, 20-32 (2012).
92. Droescher, M., Begitt, A., Marg, A., Zacharias, M. & Vinkemeier, U. Cytokine-induced paracrystals prolong the activity of signal transducers and activators of transcription (STAT) and provide a model for the regulation of protein solubility by small ubiquitin-like modifier (SUMO). *J Biol Chem* **286**, 18731-46 (2011).
93. Ungureanu, D., Vanhatupa, S., Gronholm, J., Palvimo, J.J. & Silvennoinen, O. SUMO-1 conjugation selectively modulates STAT1-mediated gene responses. *Blood* **106**, 224-6 (2005).
94. Khodarev, N.N. et al. STAT1 is overexpressed in tumors selected for radioresistance and confers protection from radiation in transduced sensitive cells. *Proc Natl Acad Sci* **101**, 1714-9 (2004).
95. Williams, C., Panaccione, R., Ghosh, S. & Rioux, K. Optimizing clinical use of mesalazine (5-aminosalicylic acid) in inflammatory bowel disease. *Therap Adv Gastroenterol* **4**, 237-48 (2011).
96. Andujar, I. et al. Inhibition of ulcerative colitis in mice after oral administration of a polyphenol-enriched cocoa extract is mediated by the inhibition of STAT1 and STAT3 phosphorylation in colon cells. *J Agric Food Chem* **59**, 6474-83 (2011).
97. Klampfer, L., Huang, J., Sasazuki, T., Shirasawa, S. & Augenlicht, L. Inhibition of interferon gamma signalling by the short chain fatty acid butyrate. *Mol Cancer Res* **1**, 855-62 (2003).
98. Tedeschi, E. et al. Green tea inhibits human inducible nitric-oxide synthase expression by down-regulating signal transducer and activator of transcription-1alpha activation. *Mol Pharmacol* **65**, 111-20 (2004).
99. Galápagos (2015) Inflammatory bowel disease - Galapagos Annual Report 2014. http://reports.glpg.com/annual-report-2014/en/r-d/inflammatory-bowel-disease.html (accessed Sep 20 2015).
100. Wagner, E.F. & Nebreda, A.R. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer* **9**, 537-49 (2009).
101. Plotnikov, A., Zehorai, E., Procaccia, S. & Seger, R. The MAPK cascades: signalling components, nuclear roles and mechanisms of nuclear translocation. *Biochim Biophys*

Acta **1813**, 1619-33 (2011).

102. Roux, P.P. & Blenis, J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* **68**, 320-44 (2004).
103. Saklatvala, J. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. *Curr Opin Pharmacol* **4**, 372-7 (2004).
104. Broom, O.J., Widjaya, B., Troelsen, J., Olsen, J. & Nielsen, O.H. Mitogen activated protein kinases: a role in inflammatory bowel disease? *Clin Exp Immunol* **158**, 272-80 (2009).
105. Cuadrado, A. & Nebreda, A.R. Mechanisms and functions of p38 MAPK signalling. *Biochem J* **429**, 403-17 (2010).
106. Roy, P.K., Rashid, F., Bragg, J. & Ibdah, J.A. Role of the JNK signal transduction pathway in inflammatory bowel disease. *World J Gastroenterol* **14**, 200-2 (2008).
107. Feng, Y.J. & Li, Y.Y. The role of p38 mitogen-activated protein kinase in the pathogenesis of inflammatory bowel disease. *J Dig Dis* **12**, 327-32 (2011).
108. Goh, K.C., Haque, S.J. & Williams, B.R. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. *EMBO J* **18**, 5601-8 (1999).
109. Zhang, Y., Cho, Y.Y., Petersen, B.L., Zhu, F. & Dong, Z. Evidence of STAT1 phosphorylation modulated by MAPKs, MEK1 and MSK1. *Carcinogenesis* **25**, 1165-75 (2004).
110. Kracht, M. & Saklatvala, J. Transcriptional and post-transcriptional control of gene expression in inflammation. *Cytokine* **20**, 91-106 (2002).
111. Reinecke, K. et al. The JNK inhibitor XG-102 protects against TNBS-induced colitis. *PLoS One* **7**, e30985 (2012).
112. Kersting, S. et al. The impact of JNK inhibitor D-JNKI-1 in a murine model of chronic colitis induced by dextran sulfate sodium. *J Inflamm Res* **6**, 71-81 (2013).
113. Coskun, M., Olsen, J., Seidelin, J.B. & Nielsen, O.H. MAP kinases in inflammatory bowel disease. *Clin Chim Acta* **412**, 513-20 (2011).
114. Kaspar, J.W., Niture, S.K. & Jaiswal, A.K. Nrf2:INrf2 (Keap1) signalling in oxidative stress. *Free Radic Biol Med* **47**, 1304-9 (2009).
115. Surh, Y.J., Kundu, J.K. & Na, H.K. Nrf2 as a master redox switch in turning on the cellular signalling involved in the induction of cytoprotective genes by some

chemopreventive phytochemicals. *Planta Med* **74**, 1526-39 (2008).

116. Kim, J., Cha, Y.N. & Surh, Y.J. A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. *Mutat Res* **690**, 12-23 (2010).
117. Naito, Y., Takagi, T., Uchiyama, K. & Yoshikawa, T. Heme oxygenase-1: a novel therapeutic target for gastrointestinal diseases. *J Clin Biochem Nutr* **48**, 126-33 (2011).
118. Dubuquoy, L. et al. PPARgamma as a new therapeutic target in inflammatory bowel diseases. *Gut* **55**, 1341-9 (2006).
119. Annese, V., Rogai, F., Settesoldi, A. & Bagnoli, S. PPARgamma in Inflammatory Bowel Disease. *PPAR Res* **2012**, 620839 (2012).
120. Varga, T., Czimmerer, Z. & Nagy, L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochim Biophys Acta* **1812**, 1007-22 (2011).
121. Glass, C.K. & Ogawa, S. Combinatorial roles of nuclear receptors in inflammation and immunity. *Nat Rev Immunol* **6**, 44-55 (2006).
122. Rousseaux, C. et al. Intestinal antiinflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator-activated receptor-gamma. *J Exp Med* **201**, 1205-15 (2005).
123. Desreumaux, P. & Ghosh, S. Review article: mode of action and delivery of 5-aminosalicylic acid - new evidence. *Aliment Pharmacol Ther* **24 Suppl 1**, 2-9 (2006).
124. Delerive, P., Fruchart, J.C. & Staels, B. Peroxisome proliferator-activated receptors in inflammation control. *J Endocrinol* **169**, 453-9 (2001).
125. Polvani, S., Tarocchi, M. & Galli, A. PPARgamma and Oxidative Stress: Con(beta) Catenating NRF2 and FOXO. *PPAR Res* **2012**, 641087 (2012).
126. Ricote, M. & Glass, C.K. PPARs and molecular mechanisms of transrepression. *Biochim Biophys Acta* **1771**, 926-35 (2007).
127. Bhattacharyya, A., Chattopadhyay, R., Mitra, S. & Crowe, S.E. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev* **94**, 329-54 (2014).
128. Piechota-Polanczyk, A. & Fichna, J. Review article: the role of oxidative stress in pathogenesis and treatment of inflammatory bowel diseases. *Naunyn Schmiedebergs Arch Pharmacol* **387**, 605-20 (2014).
129. Schieber, M. & Chandel, N.S. ROS function in redox signalling and oxidative stress. *Curr Biol* **24**, R453-62 (2014).

130. Rodrigo, R., Miranda, A. & Vergara, L. Modulation of endogenous antioxidant system by wine polyphenols in human disease. *Clin Chim Acta* **412**, 410-24 (2011).
131. Pavlick, K.P. et al. Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. *Free Radic Biol Med* **33**, 311-22 (2002).
132. Kolios, G., Valatas, V. & Ward, S.G. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology* **113**, 427-37 (2004).
133. Circu, M.L. & Aw, T.Y. Redox biology of the intestine. *Free Radic Res* **45**, 1245-66 (2011).
134. Kruidenier, L. et al. Imbalanced secondary mucosal antioxidant response in inflammatory bowel disease. *J Pathol* **201**, 17-27 (2003).
135. Kruidenier, L. et al. Differential mucosal expression of three superoxide dismutase isoforms in inflammatory bowel disease. *J Pathol* **201**, 7-16 (2003).
136. Abraham, C. & Cho, J.H. Inflammatory bowel disease. *N Engl J Med* **361**, 2066-78 (2009).
137. Morgan, M.J. & Liu, Z.G. Crosstalk of reactive oxygen species and NF-kappaB signalling. *Cell Res* **21**, 103-15 (2011).
138. Pithadia, A.B. & Jain, S. Treatment of inflammatory bowel disease (IBD). *Pharmacol Rep* **63**, 629-42 (2011).
139. Speight, R.A. & Mansfield, J.C. Drug advances in inflammatory bowel disease. *Clin Med* **13**, 378-82 (2013).
140. Triantafillidis, J.K., Merikas, E. & Georgopoulos, F. Current and emerging drugs for the treatment of inflammatory bowel disease. *Drug Des Devel Ther* **5**, 185-210 (2011).
141. Aloi, M., Nuti, F., Stronati, L. & Cucchiara, S. Advances in the medical management of paediatric IBD. *Nat Rev Gastroenterol Hepatol* **11**, 99-108 (2014).
142. Iacucci, M., de Silva, S. & Ghosh, S. Mesalazine in inflammatory bowel disease: a trendy topic once again? *Can J Gastroenterol* **24**, 127-33 (2010).
143. Karagozian, R. & Burakoff, R. The role of mesalamine in the treatment of ulcerative colitis. *Ther Clin Risk Manag* **3**, 893-903 (2007).
144. Talaei, F., Atyabi, F., Azhdarzadeh, M., Dinarvand, R. & Saadatzadeh, A. Overcoming therapeutic obstacles in inflammatory bowel diseases: a comprehensive review on novel drug delivery strategies. *Eur J Pharm Sci* **49**, 712-22 (2013).
145. Dinis, T.C., Maderia, V.M. & Almeida, L.M. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid

peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys* **315**, 161-9 (1994).

146. Ahnfelt-Ronne, I. et al. Clinical evidence supporting the radical scavenger mechanism of 5-aminosalicylic acid. *Gastroenterology* **98**, 1162-9 (1990).
147. Rowe, W. (2015) Inflammatory Bowel Disease Treatment & Management: Approach Considerations, Symptomatic Therapy/Supportive Care, Overview of Stepwise Therapy. http://emedicine.medscape.com/article/179037-treatment#d10 (accessed Sep 20 2015).
148. Carter, M.J., Lobo, A.J., Travis, S.P. & Ibd Section, B.S.o.G. Guidelines for the management of inflammatory bowel disease in adults. *Gut* **53 Suppl 5**, V1-16 (2004).
149. Fraga, C.G., Galleano, M., Verstraeten, S.V. & Oteiza, P.I. Basic biochemical mechanisms behind the health benefits of polyphenols. *Mol Aspects Med* **31**, 435-45 (2010).
150. Del Rio, D. et al. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid Redox Signal* **18**, 1818-92 (2013).
151. Martin, K.R. Polyphenols as dietary supplements: A double-edged sword. *Dovepress* **2**, 1-12 (2010).
152. Rahman, I., Biswas, S.K. & Kirkham, P.A. Regulation of inflammation and redox signalling by dietary polyphenols. *Biochem Pharmacol* **72**, 1439-52 (2006).
153. Farzaei, M.H., Rahimi, R. & Abdollahi, M. The role of dietary polyphenols in the management of inflammatory bowel disease. *Curr Pharm Biotechnol* **16**, 196-210 (2015).
154. Biasi, F., Astegiano, M., Maina, M., Leonarduzzi, G. & Poli, G. Polyphenol supplementation as a complementary medicinal approach to treating inflammatory bowel disease. *Curr Med Chem* **18**, 4851-65 (2011).
155. Manach, C., Scalbert, A., Morand, C., Remesy, C. & Jimenez, L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**, 727-47 (2004).
156. Biasi, F. et al. Wine consumption and intestinal redox homeostasis. *Redox Biol* **2**, 795-802 (2014).
157. Section, U.I.H. (2015) UNESCO Culture Sector - Intangible Heritage - 2003 Convention. http://www.unesco.org/culture/ich/RL/00884 (accessed Sep 20 2015).
158. Pervaiz, S. & Holme, A.L. Resveratrol: its biologic targets and functional activity.

Antioxid Redox Signal **11**, 2851-97 (2009).

159. Fu, Y. et al. Cyanidin-3-O-beta-glucoside ameliorates lipopolysaccharide-induced acute lung injury by reducing TLR4 recruitment into lipid rafts. *Biochem Pharmacol* **90**, 126-34 (2014).
160. Colin, D. et al. Endocytosis of resveratrol via lipid rafts and activation of downstream signalling pathways in cancer cells. *Cancer Prev Res (Phila)* **4**, 1095-106 (2011).
161. Williamson, G. & Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* **81**, 243S-255S (2005).
162. M.G., M. Anthocyanins: Antioxidant and/or anti-inflammatory activities. *Journal of Applied Pharmaceutical Science* **1**, 7-15 (2011).
163. Fang, J. Bioavailability of anthocyanins. *Drug Metab Rev* **46**, 508-20 (2014).
164. Wallace, T.C. Anthocyanins in cardiovascular disease. *Adv Nutr* **2**, 1-7 (2011).
165. McGhie, T.K. & Walton, M.C. The bioavailability and absorption of anthocyanins: towards a better understanding. *Mol Nutr Food Res* **51**, 702-13 (2007).
166. Udenigwe, C.C., Ramprasath, V.R., Aluko, R.E. & Jones, P.J. Potential of resveratrol in anticancer and anti-inflammatory therapy. *Nutr Rev* **66**, 445-54 (2008).
167. Paixao, J., Dinis, T.C. & Almeida, L.M. Dietary anthocyanins protect endothelial cells against peroxynitrite-induced mitochondrial apoptosis pathway and Bax nuclear translocation: an in vitro approach. *Apoptosis* **16**, 976-89 (2011).
168. Paixao, J., Dinis, T.C. & Almeida, L.M. Malvidin-3-glucoside protects endothelial cells up-regulating endothelial NO synthase and inhibiting peroxynitrite-induced NF-kB activation. *Chem Biol Interact* **199**, 192-200 (2012).
169. Brito, P.M. et al. Resveratrol inhibits the mTOR mitogenic signalling evoked by oxidized LDL in smooth muscle cells. *Atherosclerosis* **205**, 126-34 (2009).
170. Cianciulli, A. et al. Modulation of NF-κB activation by resveratrol in LPS treated human intestinal cells results in downregulation of PGE2 production and COX-2 expression. *Toxicol. in Vitro* **26**, 1122-1128 (2012).
171. Panaro, M.A., Carofiglio, V., Acquafredda, A., Cavallo, P. & Cianciulli, A. Anti-inflammatory effects of resveratrol occur via inhibition of lipopolysaccharide-induced NF-kappaB activation in Caco-2 and SW480 human colon cancer cells. *Br J Nutr* **108**, 1623-32 (2012).

Chapter 2

Cyanidin-3-glucoside suppresses cytokine-induced inflammatory response in human intestinal cells: comparison with 5-aminosalicylic acid

Serra D, Paixão J, Nunes C, Dinis TCP and Almeida LM (**2013**), *"Cyanidin-3-glucoside suppresses cytokine-induced inflammatory response in human intestinal cells: comparison with 5-aminosalicylic acid"*, Published in PLos ONE 8(9): e73001.

1. Abstract

The potential use of polyphenols in the prevention and treatment of chronic inflammatory diseases has been extensively investigated although the mechanisms involved in cellular signalling need to be further elucidated. Cyanidin-3-glucoside is a typical anthocyanin of many pigmented fruits and vegetables widespread in human diet. In the present study, the protection afforded by cyanidin-3-glucoside against cytokine-triggered inflammatory response was evaluated in the human intestinal HT-29 cell line, in comparison with 5-aminosalicylic acid, a well-established anti-inflammatory drug, used in inflammatory bowel disease. For this purpose, some key inflammatory mediators and inflammatory enzymes were examined. Our data showed that cyanidin-3-glucoside reduced cytokine-induced inflammation in intestinal cells, in terms of NO, PGE_2 and IL-8 production and of iNOS and COX-2 expressions, at a much lower concentration than 5-aminosalicylic acid, suggesting a higher anti-inflammatory efficiency. Interestingly, cyanidin-3-glucoside and 5-aminosalicylic acid neither prevented IkB-α degradation nor the activation of NF-kB, but significantly reduced cytokine-induced levels of activated STAT1 accumulated in the cell nucleus. In addition, we established that phosphorylated p38 MAPK was not involved in the protective effect of cyanidin-3-glucoside or 5-aminosalicylic acid. Taking into account the high concentrations of dietary anthocyanins potentially reached in the gastrointestinal tract, cyanidin-3-glucoside may be envisaged as a promising nutraceutical giving complementary benefits in the context of inflammatory bowel disease.

2. Introduction

Anthocyanins belong to the family of flavonoids and constitute the largest group of water soluble pigments in nature, responsible for the blue and purple colours of many fruits and vegetables, being consequently widespread in the human diet. Due to their relatively high consumption, the impact of anthocyanins on health promotion and disease prevention has been extensively investigated in the last decades [1-7].

Although there is some controversy regarding bioavailability of polyphenols [8, 9], they can reach concentrations up to several hundred micromolar in the gastrointestinal tract [10]. This is due in part to their abundance in the diet and also to poor intestinal absorption.

Recently, it was reported that dietary polyphenols can modulate intestinal inflammatory response, an important component of Inflammatory Bowel Disease (IBD) pathogenesis [10, 11]. IBD is a chronic and relapsing inflammatory disorder of gastrointestinal tract that includes Crohn's disease (CD) and Ulcerative Colitis (UC). In spite of its etiology remains unclear, it is believed that its occurrence is related to a genetic susceptibility of the patient to develop an exaggerated immune response to one or more promoting factors, probably commensal microorganisms present in the intestinal flora [12]. Consequently, an uncontrolled inflammation is triggered leading to tissue destruction. 5-Aminosalicylic acid (5-ASA) is a well-established drug used in adults, particularly in the treatment of mild to moderate active UC or to maintain remission periods of UC. It is known that, in most cases, 5-ASA is rapidly and extensively absorbed before reaching the colon [13]. Moreover, 5-ASA is not free of adverse effects, although it is usually well tolerated [14].

The beneficial effects of polyphenols, including anthocyanins, in humans were initially attributed to their antioxidant capacity in the prevention of diseases associated with oxidative stress, such as atherosclerosis and diabetes. Lately, some authors pointed out that other action mechanisms could be involved in the pharmacological activity of polyphenols, namely by interfering with essential signalling pathways and gene regulation [6, 7, 15, 16].

Abnormal up-regulation of nuclear factor kB (NF-kB) pathway has been observed in IBD patients and found closely related to the severity of intestinal inflammation [17]. Activation of NF-kB promotes the expression of many pro-inflammatory genes, such as those for iNOS and COX-2 [18]. However, beyond NF-kB, other transcription factors must be taken into account, such as the signal transducer and activator of transcription 1 (STAT1), whose expression and activation are heightened in IBD patients [19]. This factor also regulates the transcription of

several inflammation-associated genes, including iNOS and COX-2 [20]. Furthermore, there are many kinase pathways involved in the regulation of inflammatory response upstream transcription factors, which may also be important to unveil the mechanisms underlying the anti-inflammatory effects of polyphenols, namely p38 MAPK pathway. Actually, it has been reported that the activity of p38 MAPK is increased in patients suffering from IBD [21].

Since increasing evidences support the efficacy of anthocyanins in modulating inflammatory response [5, 11], in the present study we attempted to scrutinize the mechanisms underlying cell signalling modulation induced by a typical dietary anthocyanin, in particular cyanidin-3-glucoside (C3G) -**Figure 1A**- which is one of the most abundant anthocyanins in nature, in the presence of an inflammatory stimulus. Thus, our main goal was to assess the protection afforded by C3G against cytokine-triggered inflammatory response in the human intestinal HT-29 cell line, used as an intestinal cell model, exploring its ability to counteract the expression of crucial pro-inflammatory enzymes and pro-inflammatory mediators, in comparison with 5-ASA (**Figure 1B**).

Our data evidenced that cell pre-incubation with 25 µM C3G or 500 µM 5-ASA was effective in down-regulating the production of NO, PGE_2 and IL-8 and the expression of iNOS and COX-2 in cytokine-stimulated HT-29 cells. Interestingly, none of the compounds affected NF-kB activity. Conversely, they significantly inhibited STAT1 activation by modulating its phosphorylation. Since C3G was used in a much lower concentration than 5-ASA, C3G revealed a higher anti-inflammatory efficiency.

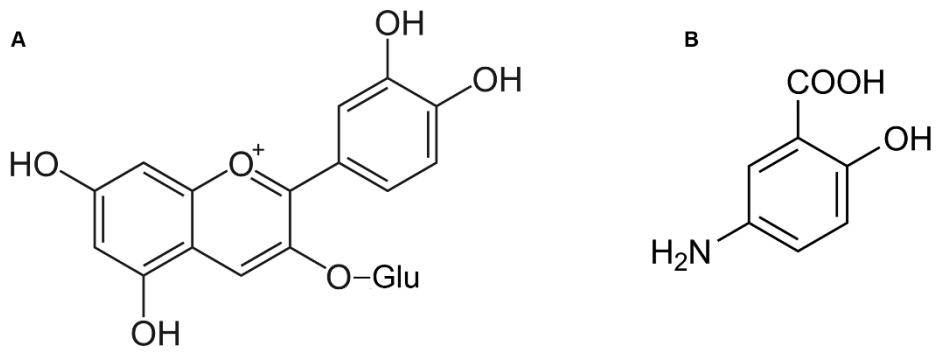


Figure 1 - Chemical structures of cyanidin-3-glucoside (A) and 5-aminosalicylic acid (B).

3. Materials and Methods

3.1 Reagents

Cyanidin-3-*o*-β-glucoside purified from natural sources was obtained from Extrasynthése (Genay, France). It had purity above 97% as measured by HPLC and was used as a solution in DMSO (5 mM) and stored in the dark, under nitrogen atmosphere, at -80ºC.

Laboratory chemicals namely dimethylsulfoxide (DMSO), sodium dodecyl sulfate (SDS), 2,3-diaminonaphthalene (DAN), 3-(4,5-dimethylthiazol-2yl)2,5-diphenyl-tetrazolium bromide (MTT), phenylmethylsulfonyl fluoride (PMSF), streptomycin/penicillin, protease inhibitor cocktail and phosphatase inhibitors were purchased from Sigma-Aldrich Co.

For cell culture, Dulbecco's modified Eagle's medium (DMEM), 0.25% trypsin, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) pH 7.4, were obtained from Gibco-Invitrogen.

Rabbit polyclonal antibody to iNOS and goat polyclonal antibody to phospho-STAT1 (Tyr701) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit polyclonal antibody to COX-2 was purchased from Abcam (Cambridge, UK); rabbit polyclonal antibody to IκB-α and rabbit monoclonal antibody to phospho-p38 MAPK (Thr180/Tyr182) were purchased from Cell Signalling Technology (MA, USA); mouse monoclonal antibody to β-actin was purchased from Sigma-Aldrich Co and anti-rabbit, anti-mouse and anti-goat IgG secondary antibodies were obtained from Abcam (Cambridge, UK).

IL-1α, TNF-α and IFN-γ were purchased from Invitrogen (NY, USA).

3.2 Cell Culture

Human colon cancer cell line (HT-29) was obtained from European Collection of Cell Cultures (Porton Down, Salisbury, UK). Cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37ºC in a humidified atmosphere of 5% CO_2. Cells were sub-cultured at confluence and used between the fourth and the twentieth passage. Before each experiment, cells at 80% confluence were starved in serum-free medium for 24 hours. Growth-arrested cultures, in medium without FBS, were treated according to the various experimental purposes.

HT-29 cells were stimulated with a cocktail of cytokines consisting of 10 ng/ml IL-1α, 20 ng/ml TNF-α and 60 ng/ml IFN-γ. Each cytokine was previously diluted in PBS with 1% BSA

and then added to cells when convenient. Cells were pre-treated with C3G, 5-ASA or both for 1 hour before exposure to the cytokines and then maintained with the inflammatory stimulus for different time intervals, depending on the assay.

3.3 Cell Viability

Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2yl) 2,5-diphenyltetrazolium bromide (MTT) to formazan, which is directly proportional to the number of living cells. After incubation for 24 hours with C3G and/or 5-ASA, $0.8x10^6$ cells/well in 6-well plates were washed with PBS and incubated with MTT (0.5 mg/ml) for 1 hour, at 37ºC. Then, the medium was removed and the formazan crystals were dissolved in DMSO (900 µl). The extent of formazan formation was recorded at 530 nm in a Synergy HT plate reader.

Results were expressed as a percentage of control cells, *i.e.* non-treated cells.

3.4 Measurement of Nitric Oxide Production

Nitric oxide production, in intestinal cells, was determined by measuring the amount of nitrite accumulated in cell culture supernatants. Nitrite was measured using a sensitive fluorimetric assay based upon the reaction of nitrite with 2,3-diaminonaphthalene (DAN), under acidic conditions, to form the fluorescent product 1-(H)-naphthotriazole [22]. Briefly, at the end of the incubation times, the supernatants were collected and nitrite was evaluated by adding 200 µl of freshly prepared DAN (0.025 mg/ml in 0.62 M HCl) to 200 µl of supernatant and mixed immediately. After 10 minutes incubation at room temperature in the dark, the reaction was stopped with 100 µl of 3 M NaOH. A standard curve was produced with known concentrations of sodium nitrite. Fluorescence intensity was read in a dual wavelength spectrophotofluorimeter, with excitation and emission at 365 nm and 405 nm, respectively. The sensitivity of the assay is 10 nM.

3.5 Assessment of Prostaglandin E_2 and IL-8 Production

Confluent HT-29 cells grown on six-well plates (0.8×10^6cells/well) were treated as above. After 16 hours of incubation, supernatants were collected and processed for PGE_2 and IL-8 quantification, by using a competitive immunoassay kit (PGE_2 EIA Kit) from Enzo Life Science

and an Elisa Kit from RayBiotech, Inc, respectively, according to the manufacturer's instructions. The values were reported to protein content as measured by the Bradford assay (Bio-Rad, USA).

3.6 Western-blot Analysis

Total, cytoplasmic and nuclear cellular protein extracts from several experiments were prepared and analysed by Western-blotting. For total cellular protein extracts, washed cell pellets were resuspended in an ice-cold lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% (w/v) glycerol, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 1 mM PMSF, 1/100 (v/v) protease inhibitor cocktail) for 20 minutes, on ice. Cell debris was subsequently removed by centrifugation at 14000 rpm for 20 minutes at 4ºC and supernatants were then collected and stored at -20ºC. Cytoplasmic protein extracts were collected essentially in the same way. Washed cells were lysed in an ice-cold buffer containing 10 mM Tris–HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 and 1% protease inhibitor cocktail, pH 7.5, for 5 minutes on ice. Afterwards, lysates were centrifuged at 5000 rpm for 5 minutes at 4ºC and the supernatants (cytoplasmic extracts) were collected and stored at -20ºC. For nuclear cellular protein extracts, the pellets were collected and resuspended in an ice-cold buffer with 20 mM Hepes, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 300 mM NaCl, 20% (w/v) glycerol, and 1% protease inhibitor cocktail, pH 7.5 and left on ice for 30 minutes. Then, lysates were centrifuged at 14000 rpm for 20 minutes at 4ºC and the supernatants (nuclear extracts) were saved at -80ºC.

Protein concentration was determined by using the Bio-Rad protein assay reagent (Bradford assay), according to the manufacturer's specifications (Bio-Rad, USA).

A range of 30-80 micrograms of reduced and denatured proteins were separated by SDS/PAGE electrophoresis on a 10% - 12% (v/v) acrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, UK) by electroblotting. To avoid non-specific binding, membranes were blocked with skimmed milk in TBS buffer supplemented with 0.1% (v/v) Tween 20 (TBS-T: 20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) and then probed with antibodies against iNOS, COX-2 and IκB-α, overnight at 4ºC and against phospho-STAT1 and phospho-p38 MAPK, 3 hours at room temperature, with a constant low shaking. Membranes were washed three times and then incubated with alkaline phosphatase-conjugated secondary antibodies (2 hours, room temperature, constant shaking). Immunoreactive complexes were detected by fluorescence in a

Typhoon 9000 scanner (Amersham Biosciences). β-Actin was used as control for protein loading. Bands were analysed using the ImageQuant TM software from Amersham Biosciences.

3.7 Evaluation of NF-kB (p65) Activity

DNA-binding activity of NF-kB-p65 was measured in nuclear extracts using the TransAM^{TM} NF-kB-p65 protein assay (Active Motif, CA, USA), an ELISA-based method with high sensitivity and reproducibility.

For preparation of nuclear extracts, washed cells were lysed in an ice-cold buffer containing 10 mM Tris–HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 and 1% protease inhibitor cocktail, pH 7.5, for 5 minutes on ice. Afterwards, lysates were centrifuged at 5000 rpm for 5 minutes at 4ºC and the supernatants (cytoplasmic extracts) were collected and stored at -20ºC. The pellets were collected and resuspended in 50 µl of Complete Lysis Buffer (a solution provided by Active Motif, CA) and left on ice for 30 minutes. Then, lysates were centrifuged at 14000 rpm for 20 minutes at 4ºC and the supernatants (nuclear extracts) were saved at -80ºC.

DNA binding activity of p65 was evaluated with 15 µg of nuclear protein, according to the manufacturer's protocol and the results expressed in relative terms.

3.8 Statistical Analysis

All data were expressed as means ± SEM of at least 3 independent assays, each one in duplicate. Differences between groups were analysed by one-way analysis of variance (ANOVA), Tukey's was used as appropriate. Values of $p<0.05$ were accepted as statistically significant.

4. Results

4.1 C3G and/or 5-ASA did not affect cell viability of HT-29 cells

In order to assess the cytotoxic effect of C3G and 5-ASA, a MTT assay was performed upon 24 hours of cell incubation with the compounds. As illustrated in **Figure 2**, neither C3G, in the concentration range of 12.5 to 50 µM, nor 500 µM 5-ASA alone or in combination with 25 µM C3G, affected the percentage of cell viability relative to the control (cells without the compounds). In contrast, the cytokines, at the concentrations used as a cell stimulus, induced a decrease of cell viability to about 50 per cent (data not shown).

In the present study, both C3G (25 µM) and 5-ASA (500 µM) were used at subtoxic concentrations.

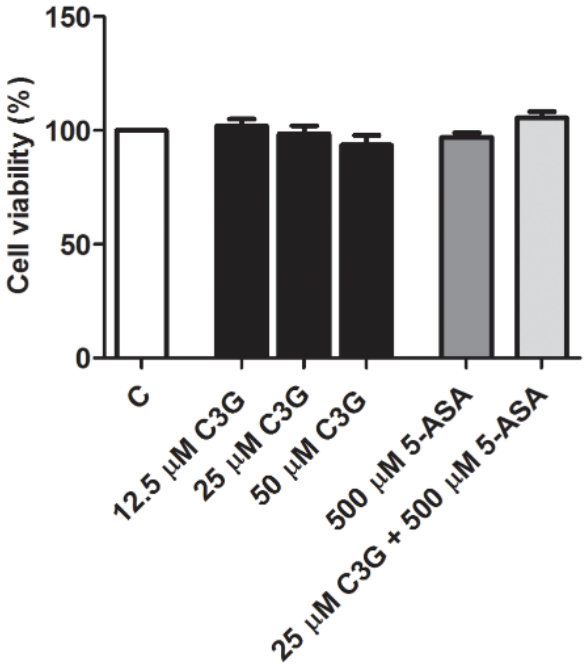


Figure 2 - Effects of C3G and 5-ASA on the cell viability of HT-29 cells. HT-29 cells were incubated for 24 hours with different concentrations of C3G (12.5 to 50 µM), 5-ASA (500 µM) and a combination of 25 µM C3G and 500 µM 5-ASA. Cell viability was assessed by MTT reduction and determined as percentage of control cells (without compounds). Values are mean ± SEM of at least three different experiments, in duplicate.

4.2 C3G inhibited secretion of pro-inflammatory mediators induced by the cytokines more efficiently than 5-ASA, in HT-29 cells

In order to evaluate the ability of C3G to inhibit pro-inflammatory mediators' production, the levels of NO, PGE_2 and IL-8 generated by cytokine-stimulated HT-29 cells were monitored.

As shown in **Figure 3A**, stimulation of HT-29 cells with cytokines, for 24 hours, induced a strong cellular nitrite formation as compared to basal values found in non-stimulated cells. Treatment with 25 µM C3G, 500 µM 5-ASA or both, for 1 hour, before cytokine stimulation, significantly reduced the nitrite levels by about 75%. Although 25 µM C3G seems to be more efficient than 500 µM 5-ASA, the difference was not significant. Combination of the two compounds (25 µM C3G and 500 µM 5-ASA) caused no further effect. Previous studies of time-dependent release of NO demonstrated that until 16 hours of incubation there was no significant cellular nitrite formation.

To examine whether C3G, 5-ASA, or the combination of both, inhibited PGE_2 and IL-8 production, cells were treated with/without the compounds for 1 hour and then treated with the cytokine mixture (IL-1α, TNF-α and IFN-γ) for 16 hours. In **Figure 3B**, it is clear that PGE_2 production was enhanced in response to cytokine treatment and that this increase was strongly inhibited by C3G by almost 65%, a higher inhibitory effect than that induced by 5-ASA (about 50%). However, no additional significant effect was observed by the combination of C3G and 5-ASA.

Likewise, IL-8 production was deeply increased by the cytokines, but only C3G was able to significantly inhibit this production. In fact, as evidenced in **Figure 3C**, the presence of 5-ASA did not suppress the IL-8 production, contrary to C3G, which led to a decrease in its production by about 20%. The combination of C3G with 5-ASA did not improve the suppressive effect, which was similar to that of C3G alone.

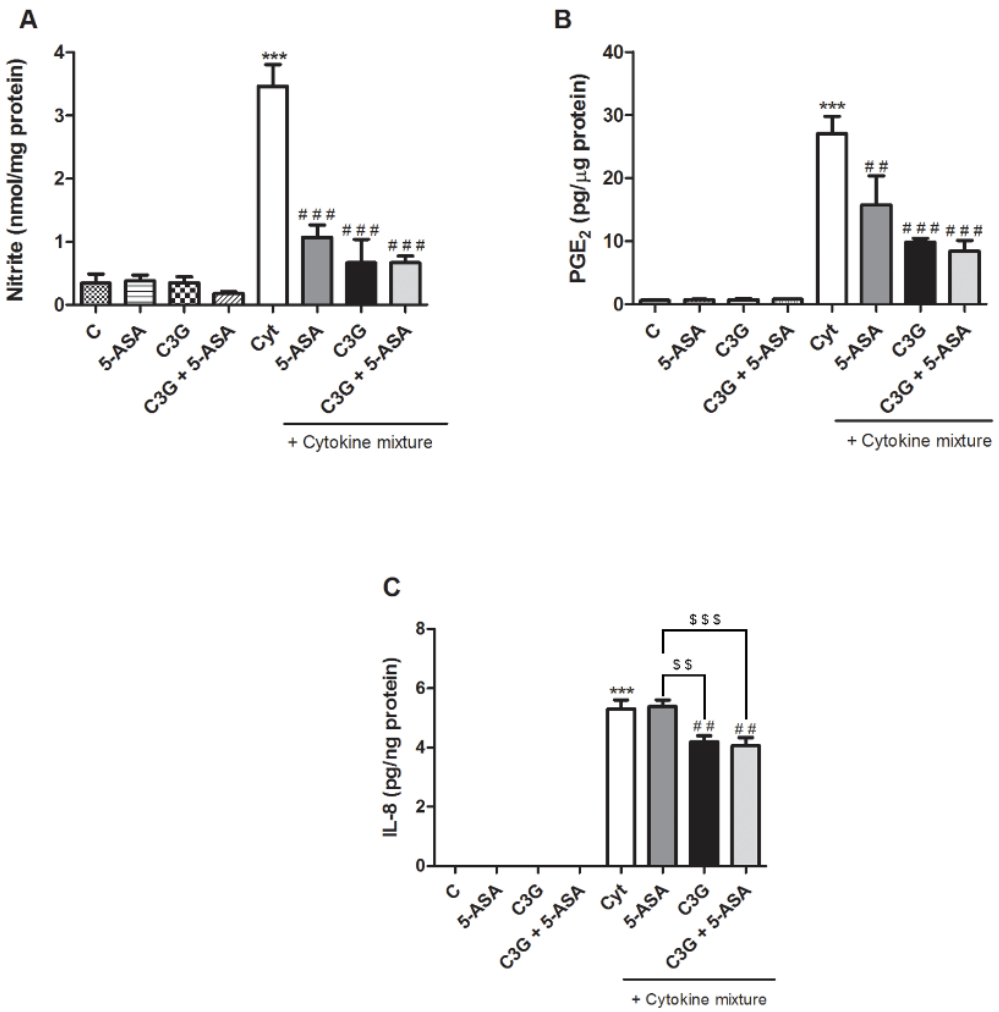


Figure 3 - C3G and 5-ASA inhibit the production of pro-inflammatory mediators induced by cytokines in HT-29 cells. Cells were pre-incubated with 25 µM C3G or 500 µM 5-ASA or both (25 µM C3G plus 500 µM 5-ASA) and then treated with cytokines for a certain period of time. NO (A), PGE_2 (B) and IL-8 (C) production by cells were measured as described in "Materials and Methods". Values are mean ± SEM of at least three different experiments, in duplicate. $^{***}P<0.001$ vs Control, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs Cytokines, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$ vs 5-ASA plus Cytokine mixture.

4.3 C3G, like 5-ASA, inhibited cytokine-induced up-expression of iNOS and COX-2 in HT-29 cells

In order to assess whether the C3G or 5-ASA-induced decrease in the pro-inflammatory mediators levels, observed into the cell culture media, was exerted via inhibition of the inducible forms of NO synthase and cyclooxygenase, protein expressions of these enzymes were determined by Western-blotting. As shown in Figure 4, in non-stimulated cells, the expression levels of iNOS (**Figure 4A**) and COX-2 (**Figure 4B**) were very low or undetectable. However, in response to cytokine stimulation and after 24 hours or 16 hours, the levels of iNOS and COX-2, respectively, were up-regulated. When cells were pre-treated with the compounds in study their expression was significantly reduced. Worth of notice is the difference in the concentrations used in this work, *i.e.*, 25 µM C3G and 500 µM 5-ASA. These concentrations were previously tested by some of us, in other studies, either with anthocyanins or with 5-ASA [6, 23]. Thus, as shown in **Figure 4A** the inhibitory effect of C3G is slightly smaller than that of 5-ASA, this is not relevant bearing into consideration the highest concentration of 5-ASA (20 times).

Regarding to COX-2 expression (**Figure 4B**), C3G more efficiently down-regulated COX-2 expression than 5-ASA and the combination of C3G and 5-ASA afforded a much better protection than the individual compounds.

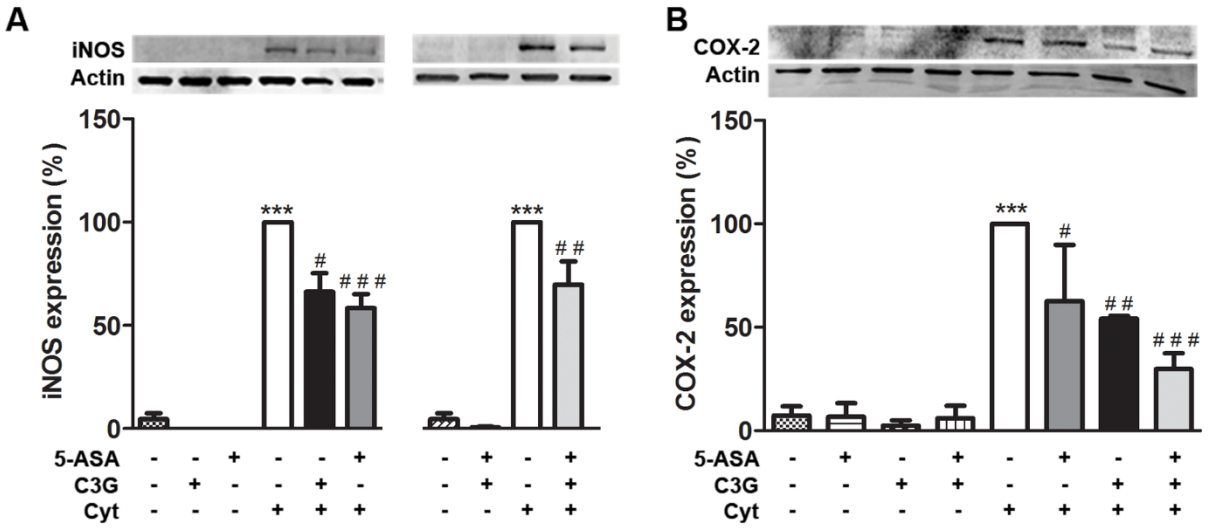


Figure 4 - C3G inhibits cytokines-induced up-expression of iNOS and COX-2, like 5-ASA, in HT-29 cells. Cells were pre-incubated with 25 µM C3G or 500 µM 5-ASA or both (25 µM C3G plus 500 µM 5-ASA) and then treated with a combination of cytokines. iNOS (A) and COX-2 (B) expressions were evaluated after 24 hours or 16 hours, respectively, in total extracts by Western blotting, as described in "Materials and Methods", and expressed as percentage of control. Values are mean ± SEM of at least three different experiments, in duplicate. $^{***}P<0.001$ vs Control, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs Cytokines.

4.4 C3G and/or 5-ASA did not inhibit cytokine-induced NF-kB activation in HT-29 cells

In an attempt to clarify the biochemical mechanism underlying the activation of iNOS, COX-2 and IL-8 observed in cytokine-stimulated HT-29 and the corresponding protection in pre-treated cells with C3G and/or 5-ASA, we evaluated the putative effects on NF-kB activation. As shown in **Figure 5**, cells stimulation for 30 minutes led to a decrease of cytoplasmic IkB-α by about 70% of the control, *i. e.* non stimulated cells. Some decrease was observed as soon as 15 minutes (data not shown), but more intensely at 30 minutes. However, cells pre-incubation with C3G and/or 5-ASA did not hamper cytokine-induced IkB-α degradation.

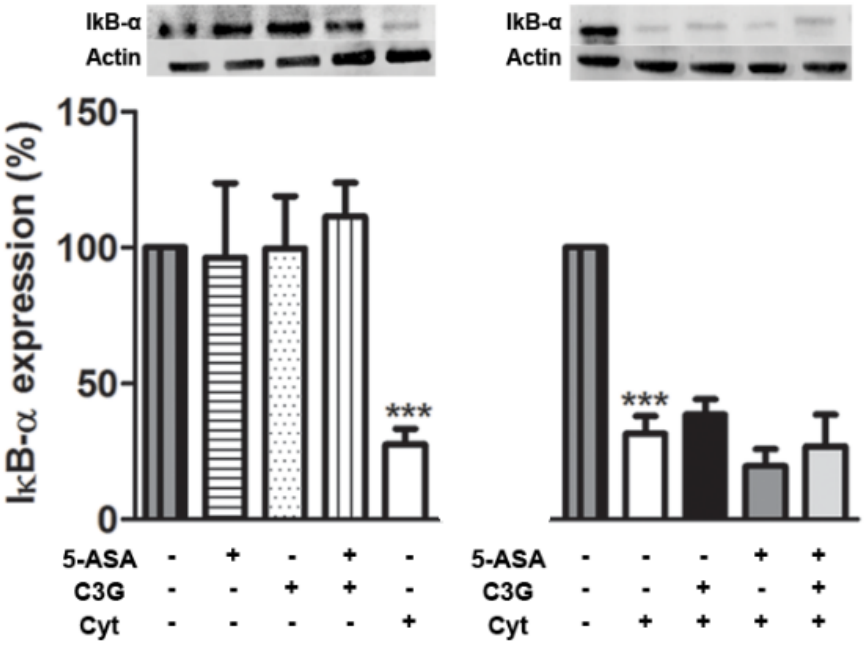


Figure 5 - Neither C3G nor 5-ASA prevent the cytokine-induced IkB-α degradation in HT-29 cells. Cells were pre-incubated with 25 µM C3G or 500 µM 5-ASA or both and then treated with a combination of cytokines for 30 minutes. IkB-α degradation was evaluated in cytoplasmic extracts by Western blotting, as described in "Materials and Methods". Values are mean ± SEM of at least three different experiments, in duplicate. $^{***}P<0.001$ vs Control.

Given that p65 accumulation and DNA binding in the cell nucleus is critical in regulating the expression of target genes, we decided to determine whether the down-regulation of iNOS and COX-2 expression and the inhibition of pro-inflammatory mediators' production, by the compounds under study, would be due to the suppression of NF-kB transcriptional activation. To test this hypothesis, cells were treated as referred and the DNA binding activity of p65 was measured, as described in materials and methods. As shown in **Figure 6**, although cells stimulation with cytokines increased the DNA binding activity of p65 up to approximately 4-fold, neither C3G nor 5-ASA alone or in combination interfered with p65 transcriptional activity, in our assay conditions.

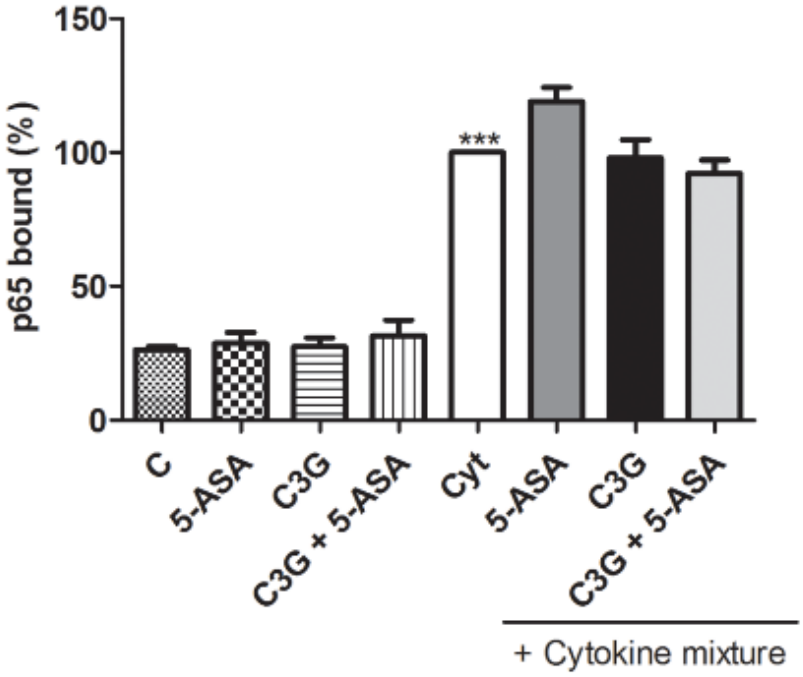


Figure 6 - C3G and 5-ASA do not suppress the activation of NF-kB-p65 in HT-29 cells. Cells were pre-incubated with 25 µM C3G or 500 µM 5-ASA or both and then treated with a combination of cytokines for 30 minutes. NF-kB activation was evaluated in nuclear extracts by a DNA-binding activity assay. Values are mean ± SEM of at least three different experiments, in duplicate. $^{***}P<0.001$ vs Control.

4.5 C3G and/or 5-ASA reduced the levels of cytokine-induced phosphorylated STAT1 in the nucleus of HT-29 cells

Considering that STAT1 is another important transcription factor that may be behind the protective effect of C3G and/or 5-ASA, we further examined the effect of these compounds on the levels of phosphorylated (activated) STAT1, in the cell nucleus. For this purpose, a Western-blotting analysis was carried out. As well as in the activation time course of NF-kB, cytokine-induced phosphorylation of STAT1 started at 15 minutes after the insult but became much stronger at 30 minutes (data not shown). It is worthwhile to note that, as illustrated in **Figure 7**, the pre-incubation with either C3G, 5-ASA or both led to a decrease in the nuclear content of this activated transcription factor, in about 50%. As happened with the other targets studied, the combination of C3G and 5-ASA did not show more benefit than the compounds alone.

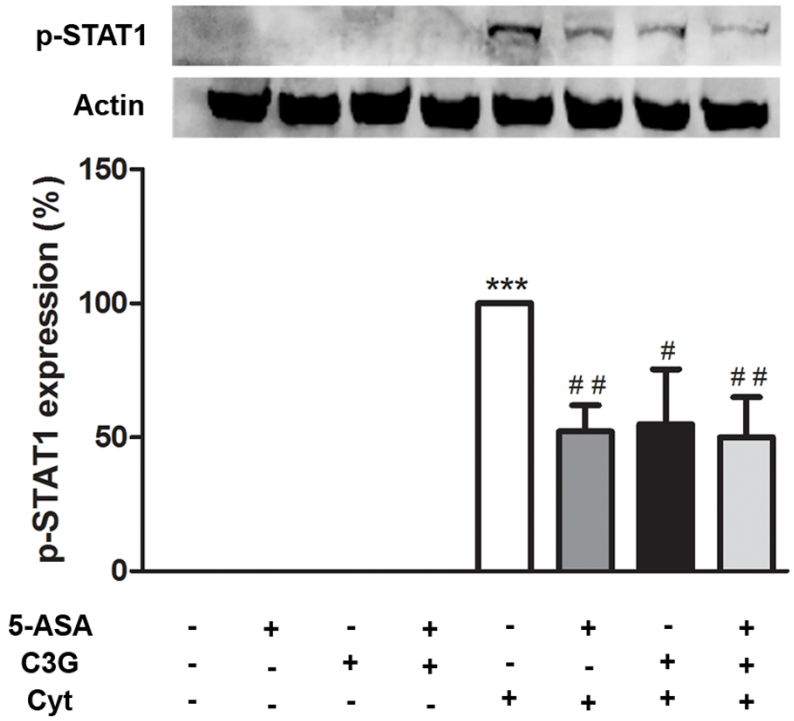


Figure 7 - C3G and 5-ASA reduce the levels of cytokine-induced STAT1 activation in HT-29 cells. Cells were pre-incubated with 25 µM C3G or 500 µM 5-ASA or both and then treated with a combination of cytokines for 30 minutes. STAT1 phosphorylation was evaluated in nuclear extracts by Western blotting, as described in "Material and Methods". Values are mean ± SEM of at least three different experiments, in duplicate. $^{***}P<0.001$ vs Control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs Cytokines.

4.6 C3G, like 5-ASA, did not affect the cytokine-induced phosphorylation of p38 MAPK, in HT-29 cells

Given that many transcription factors can be phosphorylated and activated by upstream kinases, like p38 MAPKs, it became important to explore whether anti-inflammatory action of C3G and 5-ASA was mediated through the p38 MAPK pathway in HT-29 cells. Thus, we next analysed the ability of C3G and 5-ASA to inhibit the cytokine-induced phosphorylation of p38 MAPK. Treatment of cells with the mixture of cytokines significantly induced the phosphorylation of p38 MAPK at 30 minutes. However, at this time, C3G and/or 5-ASA did not counteract this process, as it is shown by Western-blotting technique (**Figure 8**).

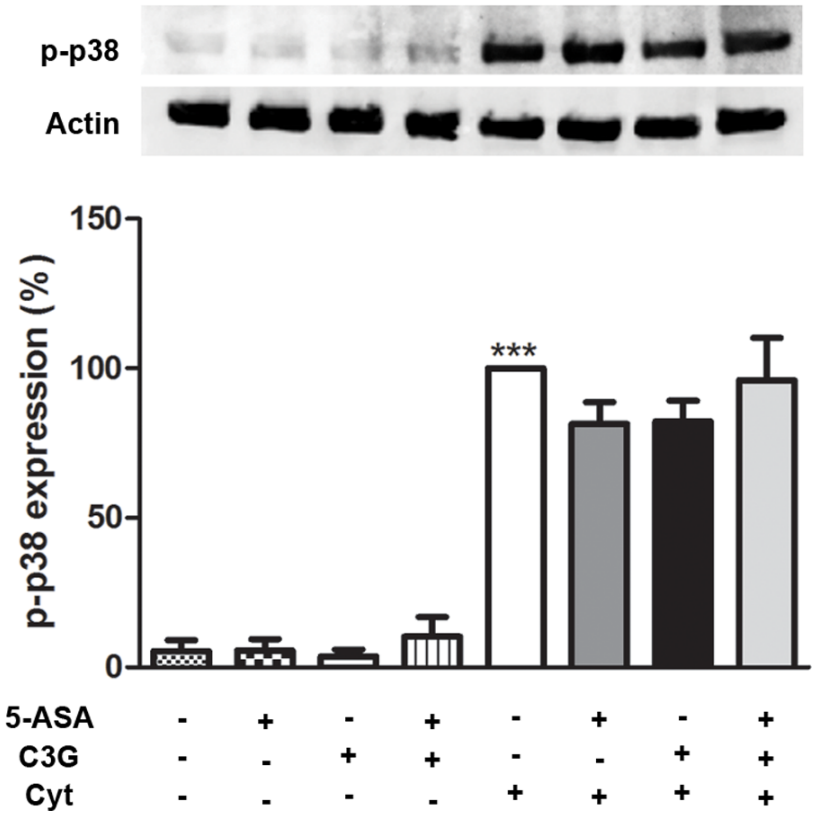


Figure 8 - Failure of C3G and 5-ASA to inhibit cytokine-induced phosphorylation of p38 MAPK in HT-29 cells. Cells were pre-incubated with 25 µM C3G or 500 µM 5-ASA or both and then treated with a combination of cytokines for 30 minutes. Phosphorylation of p38 MAPK was evaluated in total extracts by Western blotting, as described in "Material and Methods". Values are mean ± SEM of at least three different experiments, in duplicate. $^{***}P<0.001$ vs Control.

5. Discussion

In the last years, many studies have been carried out demonstrating the important role of anthocyanins in the prevention and treatment of chronic inflammatory diseases [5, 7]. It is known that health effects of polyphenols depend on the ingested amount and on the achieved bioavailability which deeply differs among the various polyphenols [8]. In what concerns anthocyanins, it is believed that their bioavailability is low, but it is also known that these compounds are unique because they can exist in many different molecular forms, in a dynamic equilibrium, and the currently used analytical methods underestimate the bioavailability data [2, 9]. Apart from this, many studies confirm that anthocyanins can be very active at the intestine level, reaching high concentrations in the gastrointestinal tract, which may be explained by their abundance in diet and poor absorption [8, 9]. This fact makes anthocyanins very attractive for exploring their anti-inflammatory potential in IBD context, in particular that of cyanidin-3-glucoside (C3G), one of the most abundant anthocyanins in nature.

The present study was undertaken to provide new insights into cellular signalling mechanisms underlying the ability of C3G to protect intestinal cells (HT-29), against pro-inflammatory stimulus, in comparison with 5-aminosalicylic acid (5-ASA). A combination of cytokines containing IL-1α, TNF-α, and IFN-γ was selected as a pro-inflammatory stimulus, since it is known that these cytokines are rapidly released by injured tissue or infection and are effective as inducers of the expression of different pro-inflammatory genes, depending on the cell type [24-26].

Thus, in the intestinal cell line used in this work, C3G was able to efficiently inhibit the NO production (Figure 3A) counteracting the iNOS expression (Figure 4A) like 5-ASA, but at a much lower concentration. NO is a free radical produced from the amino acid L-arginine by nitric oxide synthase (NOS) enzyme. Although NO, in constitutive levels, has a physiological role in maintaining adequate perfusion and regulation of microvascular and epithelial permeability [27], persistent overproduction of NO via up-regulation of iNOS is associated with inflammatory response leading to gut barrier injury [28]. The protective effects of C3G, with respect to iNOS and NO production, are very interesting because they revealed that this anthocyanin, which is a natural polyphenol widespread in plants, acted in a very similar way to 5-ASA, which in turn is a potent anti-inflammatory therapeutic agent, commonly used in clinical practice. Furthermore, C3G acted in a concentration 20 times lower than 5-ASA. Nonetheless, the combination of the two compounds did not provide better protection over the

individual compounds, excluding either additive or synergistic effects.

In contrast to the effect on iNOS, C3G/5-ASA combination demonstrated a higher protection for COX-2 expression than that provided by the individual compounds (Figure 4B). COX-2 is the inducible form of COX and its expression is up-regulated in the inflamed gut of IBD patients [29]. Thus, high levels of prostaglandins have also been found in the mucosa of IBD patients [29] leading to the perpetuation of inflammation [30]. In this way, we also proved the ability of C3G to prevent PGE_2 biosynthesis and in agreement with COX-2 results, C3G inhibited PGE_2 production more significantly than 5-ASA (Figure 3B), bearing into consideration the much lower concentration of C3G used to achieve this inhibitory effect. However, the combined effects of C3G plus 5-ASA on PGE_2 production were similar to that of C3G, revealing no advantage in adding 5-ASA over C3G. Also, of note is that this cell line expresses mRNA for IL-8 and secretes it after stimulation [31]. Actually, we observed such overproduction, but only C3G pre-treatment was able to significantly reduce it (Figure 3C).

Although it is known that the transcriptional regulation of iNOS and COX-2 is complex [32, 33], the nuclear factor kB is one of the most important regulators of pro-inflammatory genes expression and it is well-established that its activation is significantly induced in intestine of IBD patients [17, 34]. So, the effects observed in pre-treated HT-29 cells with either C3G or 5-ASA could be due to the suppression of NF-kB activation. This activation process can be initiated by a wide variety of different stimuli, which lead to the phosphorylation and degradation of the NF-kB inhibitory molecules, IkB proteins [17]. In the present study, we observed that in our assay conditions the combination of cytokines was able to induce the degradation of IkB-α but, unexpectedly, neither C3G nor 5-ASA inhibited such degradation (Figure 5). This degradative process is a crucial step for the activation of NF-kB with subsequent translocation to the nucleus and binding to DNA, in the classical pathway of NF-kB activation. However, recent evidences indicate that transcriptional activity of NF-kB also requires the direct modification of NF-kB proteins, namely by phosphorylation and acetylation. The loss of phosphorylation of p65 interferes with its DNA binding and transactivation activities [18, 35-37]. Taking this into account and considering that other authors have already reported the inhibitory effect of 5-ASA on inducible NF-kB-dependent transcription in intestinal epithelial cells, independent of preventing the IkB-α degradation [38], we investigated whether C3G or 5-ASA or both could hamper NF-kB activation, by interfering with its DNA binding. However, in our assay conditions, in cells pre-treated with the compounds no prevention of NF-kB activation was observed (Figure 6). Our findings seem to conflict with

those of Min *et al* [39], who have reported an inhibitory effect of C3G on LPS-induced NF-kB activation in RAW 264.7 cells. These contradictory results might be explained by the differences in the cell type and in the pro-inflammatory stimulus.

Thus, our belief is that the anti-inflammatory effects of C3G observed in HT-29 cells, stimulated by a cocktail of cytokines, could be related to the suppression of an alternative cell signalling, other than NF-kB. This is consistent with reports by others showing that some polyphenols preferentially suppress STAT1 activation rather than NF-kB activation [40, 41]. The JAK-STAT signalling pathway is a common signalling pathway activated by various stimuli, namely interferons. The binding of a cytokine to its cell-surface receptor results in the activation of JAK tyrosine kinases, which in turn phosphorylate STATs. Then, STATs dimerize, translocate into the nucleus and activate the transcription of STAT-responsive genes, namely iNOS [42-45]. The present study demonstrated, for the first time, that pre-incubation of C3G in HT-29 cells decreased the nuclear levels of this activated transcription factor, to about 50% (Figure 7). A similar effect was obtained by 5-ASA but at a much higher concentration than that of C3G. One possible explanation to such decrease in nuclear activated STAT1 levels could be the induction of the expression of the SOCS family of proteins by our compounds. These proteins are in part in charge of the negative feedback mechanism engaged by STATs. They can block the recruitment of STATs, bind to JAKs, or even target STATs for proteasomal degradation [42, 43, 46]. On the other hand, another possible explanation will be that C3G and 5-ASA can induce the dephosphorylation of STAT1. Actually, it has been described that tyrosine-phosphorylated STAT1 requires to be dephosphorylated, by some nuclear phosphatases, in order to leave the nucleus [43, 47].

Furthermore, there are many kinase pathways involved in the regulation of inflammatory response upstream transcription factors, which may also be important to understand the mechanisms behind the anti-inflammatory effects of C3G, namely the p38 MAPK pathway. In fact, this pathway has already been identified as crucial for induction of iNOS and COX-2 in HT-29 cells by a mixture of cytokines [48]. However, under our experimental conditions none of the compounds was successful in inhibiting the phosphorylation (activation) of p38 MAPK, revealing that p38 MAPK was not involved in the protective effect of C3G and 5-ASA.

In conclusion, *in vitro*, under our experimental conditions, C3G showed to be effective in inhibiting cytokine-induced pro-inflammatory markers, namely NO, PGE_2, IL-8, iNOS and COX-2, without affecting the activation of either NF-kB or p38 MAPK but significantly decreasing the amount of activated STAT1 in the nucleus, in HT-29 cells. Moreover, we

demonstrated, for the first time, that in comparison with 5-ASA, C3G has a stronger anti-inflammatory activity regarding the studied pro-inflammatory markers in particular, taking into account the difference in the concentrations used. However, it is known that, *in vivo*, anthocyanins suffer from spontaneous degradation and are metabolized by the indigenous microbiota population in the colon [49] which causes the release of aglycones of their glycosides and eventually the disruption of the ring [49, 50]. Thus, unanswered questions should lead to interesting future research to clarify the actual molecular structures underlying the protective effects of C3G and also to investigate the accessibility of them to the epithelium.

Despite this and considering that current treatment options in patients with IBD are not curative and patients face lifelong therapy, C3G may be envisaged as a promising nutraceutical giving complementary benefits in attenuating inflammation and decreasing the risk for the development of colorectal cancer observed in these patients.

6. References

1. Scalbert, A., Manach, C., Morand, C., Remesy, C. & Jimenez, L. Dietary polyphenols and the prevention of diseases. *Crit Rev Food Sci Nutr* **45**, 287-306 (2005).
2. Prior, R.L. & Wu, X. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic Res* **40**, 1014-28 (2006).
3. Wang, L.S. & Stoner, G.D. Anthocyanins and their role in cancer prevention. *Cancer Letters* **269**, 281-290 (2008).
4. He, J.A. & Giusti, M.M. Anthocyanins: Natural Colorants with Health-Promoting Properties. *Annual Review of Food Science and Technology, Vol 1* **1**, 163-187 (2010).
5. M.G., M. Anthocyanins: Antioxidant and/or anti-inflammatory activities. *Journal of Applied Pharmaceutical Science* **1**, 7-15 (2011).
6. Paixao, J., Dinis, T.C. & Almeida, L.M. Dietary anthocyanins protect endothelial cells against peroxynitrite-induced mitochondrial apoptosis pathway and Bax nuclear translocation: an in vitro approach. *Apoptosis* **16**, 976-89 (2011).
7. Paixao, J., Dinis, T.C. & Almeida, L.M. Malvidin-3-glucoside protects endothelial cells up-regulating endothelial NO synthase and inhibiting peroxynitrite-induced NF-kB activation. *Chem Biol Interact* **199**, 192-200 (2012).
8. Manach, C., Scalbert, A., Morand, C., Remesy, C. & Jimenez, L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**, 727-47 (2004).
9. McGhie, T.K. & Walton, M.C. The bioavailability and absorption of anthocyanins: towards a better understanding. *Mol Nutr Food Res* **51**, 702-13 (2007).
10. Romier, B., Schneider, Y.J., Larondelle, Y. & During, A. Dietary polyphenols can modulate the intestinal inflammatory response. *Nutr Rev* **67**, 363-78 (2009).
11. Piberger, H. et al. Bilberries and their anthocyanins ameliorate experimental colitis. *Mol Nutr Food Res* **55**, 1724-9 (2011).
12. Khor, B., Gardet, A. & Xavier, R.J. Genetics and pathogenesis of inflammatory bowel disease. *Nature* **474**, 307-17 (2011).
13. Crotty, B. & Jewell, D.P. Drug therapy of ulcerative colitis. *Br J Clin Pharmacol* **34**, 189-98 (1992).
14. Stein, R.B. & Hanauer, S.B. Comparative tolerability of treatments for inflammatory bowel disease. *Drug Saf* **23**, 429-48 (2000).
15. Mauray, A. et al. Nutrigenomic analysis of the protective effects of bilberry

anthocyanin-rich extract in apo E-deficient mice. *Genes Nutr* **5**, 343-53 (2010).

16. McCune, L.M., Kubota, C., Stendell-Hollis, N.R. & Thomson, C.A. Cherries and health: a review. *Crit Rev Food Sci Nutr* **51**, 1-12 (2011).
17. Atreya, I., Atreya, R. & Neurath, M.F. NF-kappa B in inflammatory bowel disease. *J Intern Med* **263**, 591-596 (2008).
18. Li, Q. & Verma, I.M. NF-kappaB regulation in the immune system. *Nat Rev Immunol* **2**, 725-34 (2002).
19. Schreiber, S. et al. Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. *Gut* **51**, 379-85 (2002).
20. Yoshimura, A. Signal transduction of inflammatory cytokines and tumor development. *Cancer Sci* **97**, 439-47 (2006).
21. Feng, Y.J. & Li, Y.Y. The role of p38 mitogen-activated protein kinase in the pathogenesis of inflammatory bowel disease. *J Dig Dis* **12**, 327-32 (2011).
22. Wright, K., Ward, S.G., Kolios, G. & Westwick, J. Activation of phosphatidylinositol 3-kinase by interleukin-13. An inhibitory signal for inducible nitric-oxide synthase expression in epithelial cell line HT-29. *J Biol Chem* **272**, 12626-33 (1997).
23. Dinis, T.C., Maderia, V.M. & Almeida, L.M. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys* **315**, 161-9 (1994).
24. Nathan, C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* **6**, 3051-64 (1992).
25. Kolios, G., Brown, Z., Robson, R.L., Robertson, D.A. & Westwick, J. Inducible nitric oxide synthase activity and expression in a human colonic epithelial cell line, HT-29. *Br J Pharmacol* **116**, 2866-72 (1995).
26. Kolios, G. et al. Ciprofloxacin inhibits cytokine-induced nitric oxide production in human colonic epithelium. *Eur J Clin Invest* **36**, 720-9 (2006).
27. Kolios, G., Valatas, V. & Ward, S.G. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology* **113**, 427-37 (2004).
28. Salerno, L., Sorrenti, V., Di Giacomo, C., Romeo, G. & Siracusa, M.A. Progress in the development of selective nitric oxide synthase (NOS) inhibitors. *Curr Pharm Des* **8**, 177-200 (2002).
29. Singer, II et al. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory

bowel disease. *Gastroenterology* **115**, 297-306 (1998).

30. Sheibanie, A.F. et al. The proinflammatory effect of prostaglandin E2 in experimental inflammatory bowel disease is mediated through the IL-23-->IL-17 axis. *J Immunol* **178**, 8138-47 (2007).
31. Jijon, H.B., Panenka, W.J., Madsen, K.L. & Parsons, H.G. MAP kinases contribute to IL-8 secretion by intestinal epithelial cells via a posttranscriptional mechanism. *Am J Physiol Cell Physiol* **283**, C31-41 (2002).
32. Chun, K.S. & Surh, Y.J. Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. *Biochem Pharmacol* **68**, 1089-100 (2004).
33. Kleinert, H., Pautz, A., Linker, K. & Schwarz, P.M. Regulation of the expression of inducible nitric oxide synthase. *Eur J Pharmacol* **500**, 255-66 (2004).
34. Surh, Y.J., Na, H.K., Lee, J.Y. & Keum, Y.S. Molecular mechanisms underlying anti-tumor promoting activities of heat-processed Panax ginseng C.A. Meyer. *J Korean Med Sci* **16 Suppl**, S38-41 (2001).
35. Zhong, H., May, M.J., Jimi, E. & Ghosh, S. The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. *Mol Cell* **9**, 625-36 (2002).
36. Hoffmann, A. & Baltimore, D. Circuitry of nuclear factor kappaB signalling. *Immunol Rev* **210**, 171-86 (2006).
37. Oeckinghaus, A., Hayden, M.S. & Ghosh, S. Crosstalk in NF-kappaB signalling pathways. *Nat Immunol* **12**, 695-708 (2011).
38. Egan, L.J. et al. Inhibition of interleukin-1-stimulated NF-kappaB RelA/p65 phosphorylation by mesalamine is accompanied by decreased transcriptional activity. *J Biol Chem* **274**, 26448-53 (1999).
39. Min, S.W., Ryu, S.N. & Kim, D.H. Anti-inflammatory effects of black rice, cyanidin-3-O-beta-D-glycoside, and its metabolites, cyanidin and protocatechuic acid. *Int Immunopharmacol* **10**, 959-66 (2010).
40. Liu, P.W., Chen, M.F., Tsai, A.P. & Lee, T.J. STAT1 mediates oroxylin a inhibition of iNOS and pro-inflammatory cytokines expression in microglial BV-2 cells. *PLoS One* **7**, e50363 (2012).
41. Tedeschi, E. et al. Green tea inhibits human inducible nitric-oxide synthase expression by down-regulating signal transducer and activator of transcription-1alpha activation.

Mol Pharmacol **65**, 111-20 (2004).

42. Santos, C.I. & Costa-Pereira, A.P. Signal transducers and activators of transcription-from cytokine signalling to cancer biology. *Biochim Biophys Acta* **1816**, 38-49 (2011).
43. Shuai, K. & Liu, B. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol* **3**, 900-11 (2003).
44. Kramer, O.H. & Heinzel, T. Phosphorylation-acetylation switch in the regulation of STAT1 signalling. *Mol Cell Endocrinol* **315**, 40-8 (2010).
45. Ramana, C.V., Chatterjee-Kishore, M., Nguyen, H. & Stark, G.R. Complex roles of Stat1 in regulating gene expression. *Oncogene* **19**, 2619-27 (2000).
46. Klampfer, L. Signal transducers and activators of transcription (STATs): Novel targets of chemopreventive and chemotherapeutic drugs. *Curr Cancer Drug Targets* **6**, 107-21 (2006).
47. Haspel, R.L. & Darnell, J.E., Jr. A nuclear protein tyrosine phosphatase is required for the inactivation of Stat1. *Proc Natl Acad Sci U S A* **96**, 10188-93 (1999).
48. Paul, S. et al. Anti-inflammatory action of pterostilbene is mediated through the p38 mitogen-activated protein kinase pathway in colon cancer cells. *Cancer Prev Res* **2**, 650-7 (2009).
49. Aura, A.M. et al. In vitro metabolism of anthocyanins by human gut microflora. *Eur J Nutr* **44**, 133-42 (2005).
50. Kay, C.D., Kroon, P.A. & Cassidy, A. The bioactivity of dietary anthocyanins is likely to be mediated by their degradation products. *Mol Nutr Food Res* **53 Suppl 1**, S92-101 (2009).

Chapter 3

Resveratrol modulates cytokine-induced JAK/STAT activation more efficiently than 5-aminosalicylic acid: an in vitro approach

Serra D, Rufino AT, Mendes AF, Almeida LM and Dinis TCP (**2014**), "*Resveratrol modulates cytokine-induced JAK/STAT activation more efficiently than 5-aminosalicylic acid: an in vitro approach"*, Published in PLos ONE 9(10): e109048.

1. Abstract

Background: Many advances have been recently made focused on the valuable help of dietary polyphenols in chronic inflammatory diseases. On the other hand, current treatment options for inflammatory bowel disease patients are unsatisfying and, for this reason, it is estimated that many patients use dietary supplements to achieve extra benefits.

Aim: The aim of this work was to analyse under a mechanistic perspective the anti-inflammatory potential of resveratrol, a natural polyphenolic compound, and to compare it with a pharmaceutical agent, 5-aminosalicylic acid, using the intestinal HT-29 cell line, as a cellular model.

Methodology and Principal Findings: In the present study, HT-29 colon epithelial cells were pre-treated with 25 μM resveratrol and/or 500 μM 5-aminosalicylic acid and then exposed to a combination of cytokines (IL-1α, TNF-α, IFN-γ) for a certain period of time. Our data showed that resveratrol, used in a concentration 20 times lower than 5-aminosalicylic acid, was able to significantly reduce NO and PGE_2 production, iNOS and COX-2 expression and reactive oxidant species formation induced by the cytokine challenge. However, as already verified with 5-aminosalicylic acid, in spite of not exhibiting any effect on IkB-α degradation, resveratrol down-regulated JAK-STAT pathway, decreasing the levels of activated STAT1 in the nucleus. Additionally, resveratrol decreased the cytokine-stimulated activation of SAPK/JNK pathway but did not counteract the cytokine-triggered negative feedback mechanism of STAT1, through p38 MAPK.

Conclusion/Significance: Taken together, our results show that resveratrol may be considered a future nutraceutical approach, promoting remission periods, limiting the inflammatory process and preventing colorectal cancer, which is common in these patients.

2. Introduction

In the last decades, many studies have shed light on the impact of dietary polyphenols in chronic inflammatory diseases, namely diabetes [1-3], atherosclerosis [2, 4] and inflammatory bowel diseases [5, 6]. Although the cell signalling mechanisms involved are far from being fully understood, many recent studies believe that the consumption of these biological phytochemicals can be truly advantageous to avoid or limit diseases progression [2, 3, 7-10]. For instance, some epidemiological studies show that a moderated intake of red wine (rich in polyphenols) can be useful in the prevention of cardiovascular diseases [11].

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a natural non-flavonoid polyphenol, found mainly in grapes and red-wine and is one of the most studied polyphenols [10, 12-15]. However, the major concerns about resveratrol (Resv) efficacy are related to its low oral bioavailability [16, 17] and to the possible induction of liver damage [18]. Nevertheless, previous reports demonstrated that resveratrol has an important role as an anti-inflammatory agent [6, 14, 19, 20] and considering that the intestine is a target site for resveratrol action, it is of great interest to further understand the beneficial effects of resveratrol in an intestinal disease which is mainly characterized by inflammation, as the Inflammatory Bowel Disease (IBD). IBD is a chronic inflammatory disorder of the gastrointestinal tract, which includes Crohn's Disease and Ulcerative Colitis, characterized by periods of remission and of relapses, whose etiology remains enigmatic [21, 22]. Until now, this disease does not have cure and consequently the pharmacological treatment is used to prevent and treat symptoms and still to induce or maintain the remission periods. There has been a huge advance in the therapy options for IBD patients but the conventional therapies, as the well-known anti-inflammatory 5-aminosalicylic acid (5-ASA), remain the cornerstone of treatment for the majority of these patients [23]. Besides, since existing treatment options for IBD patients often bring marginal results, dietary supplements have deserved increasing interest to achieve extra benefits. Therefore, the advantages and implications of such dietary supplements for IBD patients need to be more elucidated. A previous work performed in our laboratory has focused on the anti-inflammatory potential of the flavonoid polyphenol, cyanidin-3-glucoside, in comparison with the active principle, 5-ASA, in the context of IBD [8]. The aim of the present study was to extend this research to a polyphenol with a completely different chemical structure, the resveratrol, and to explore, under a mechanistic perspective, its anti-inflammatory potential as compared to 5-ASA (**Figure 1**). For this purpose, the HT-29 cell line was used as a colon epithelial cells model,

stimulated by a mixture of cytokines (Cyt). Cytokines are molecules rapidly released by injured tissues and are inducers of inflammatory response [24, 25]. Some previous studies have suggested that exposure of intestinal cells to a mixture of cytokines can activate inflammatory cascades (namely, NF-kB, MAPKs and JAK-STAT pathways) and in turn increase the expression of pro-inflammatory enzymes, (iNOS and COX-2), the production of pro-inflammatory mediators (NO and PGE_2) and the formation of reactive oxygen species (ROS) [26-28]. The down-regulation of these pro-inflammatory cascades emerges as a valuable strategy in IBD, since they are usually heightened in these patients [29-32].

Our data demonstrated, for the first time, the stronger anti-inflammatory efficiency of Resv as compared to 5-ASA, given that 25 μM Resv was more effective than 500 μM 5-ASA in down-regulating the production of pro-inflammatory mediators (NO, PGE_2), pro-inflammatory enzymes (iNOS, COX-2 mRNAs and proteins) and ROS formation induced by the cytokines. Moreover, as it was previously verified by some of us for 5-ASA [8], Resv did not affect IkB-α degradation, but significantly decreased the amount of activated STAT1 in the nucleus of cytokine-stimulated HT-29 cells. Besides, Resv, alone or in combination with 5-ASA, down-regulated STAT1 activation through a dependent p38 MAPK mechanism and inhibited the cytokine-induced SAPK/JNK activation. Therefore, our work gives a step forward unravelling JAK-STAT as well as MAPK signalling as key cascades involved in Resv anti-inflammatory protection and in its potential anticancer action.

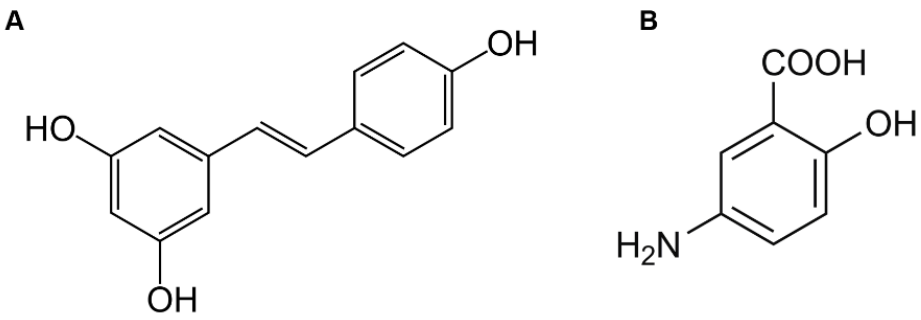


Figure 1 - Chemical structures of resveratrol (A) and 5-aminosalicylic acid (B).

3. Materials and Methods

3.1 Reagents

Resveratrol purified from natural sources was obtained from Extrasynthése (Genay, France). Its purity was above 95%, as measured by HPLC, and it was used as a solution in DMSO (5 mM) and stored at -20 ºC.

Laboratory chemicals namely dimethylsulfoxide (DMSO), sodium dodecyl sulfate (SDS), 2,3-diaminonaphthalene (DAN), 3-(4,5-dimethylthiazol-2yl)2,5-diphenyl-tetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), phenylmethylsulfonyl fluoride (PMSF), Hoechst 33258, streptomycin/penicillin, protease inhibitor cocktail and phosphatase inhibitors were purchased from Sigma-Aldrich Co.

For cell culture, Dulbecco's modified Eagle's medium (DMEM), 0.25% trypsin, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) pH 7.4, were obtained from Gibco-Invitrogen.

Rabbit polyclonal antibody to iNOS and goat polyclonal antibody to phospho-STAT1 (Tyr701) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit monoclonal antibody to phospho-p38 MAPK (Thr180/Tyr182) and rabbit polyclonal antibodies to phospho-SAPK/JNK (Thr183/Thr185) and to IκB-α were purchased from Cell Signalling Technology (MA, USA); mouse monoclonal antibodies to β-actin and to β-tubulin were purchased from Sigma-Aldrich Co and rabbit polyclonal antibodies to COX-2, to lamin B1 and anti-rabbit, anti-mouse, anti-goat IgG secondary antibodies were obtained from Abcam (Cambridge, UK). The Alexa Fluor 594 chicken anti-goat IgG fluorescent secondary antibody was bought from Alfagene (Life Technologies).

IL-1α, TNF-α and IFN-γ were purchased from Invitrogen (NY, USA).

3.2 Cell Culture

Human colon cancer cell line (HT-29) and human liver carcinoma cell line (HepG2) were obtained from European Collection of Cell Cultures (Porton Down, Salisbury, UK). Both cell lines were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37ºC in a humidified atmosphere of 5% CO_2. Cells were passaged weekly and sub-cultured at confluence. Before each experiment, cells at 80% confluence were starved in serum-free medium for 24 hours. Growth-arrested cultures, in medium without FBS, were

treated according to the various experimental purposes.

HT-29 cells were stimulated with a combination of cytokines consisting of 10 ng/ml IL-1α, 20 ng/ml TNF-α and 60 ng/ml IFN-γ. Each cytokine was previously diluted in 1% BSA in PBS. HT-29 cells were pre-treated with Resv, 5-ASA or both for 1 hour before the exposure to the cytokines and then maintained with the inflammatory stimulus for different time periods, depending on the experiment.

3.3 Cell Viability

Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2yl) 2,5-diphenyltetrazolium bromide (MTT) to formazan, which is directly proportional to the number of living cells. Cells in 6-well plates (8 x 10^5 cells/well) were pre-treated with several concentrations of Resv, 500 µM 5-ASA or 25 µM Resv plus 500 µM 5-ASA for 24 hours. At the end, cells were washed with PBS and incubated with MTT (0.5 mg/ml) for 1 hour, at 37ºC. Then, the medium was removed and the formazan crystals were dissolved in DMSO (900 µl). The extent of formazan formation was recorded at 530 nm in a Synergy HT plate reader.

Results were expressed as a percentage of control cells, *i.e.* non-treated cells.

3.4 Measurement of Nitric Oxide Production

Nitric oxide production, in intestinal cells, was determined by measuring the amount of nitrite accumulated in cell culture supernatants. Nitrite was measured using a sensitive fluorimetric assay based upon the reaction of nitrite with 2,3-diaminonaphthalene (DAN), under acidic conditions, to form the fluorescent product 1-(H)-naphthotriazole [33]. Briefly, at the end of 24 hours of incubation, the supernatants were collected and nitrite was evaluated by adding 200 µl of freshly prepared DAN (0.025 mg/ml in 0.62 M HCl) to 200 µl of supernatant and mixed immediately. After 10 minutes of incubation at room temperature in the dark, the reaction was stopped with 100 µl of 3 M NaOH. A standard curve was produced with known concentrations of sodium nitrite. Fluorescence intensity was read in a dual wavelength spectrophotofluorimeter, with excitation and emission at 365 nm and 405 nm, respectively. The sensitivity of the assay is 10 nM.

3.5 Assessment of Prostaglandin E_2 Production

Confluent HT-29 cells grown on six-well plates (8 x 10^5 cells/well) were treated as described in cell culture. After 16 hours of incubation time, supernatants were collected and processed for PGE_2 quantification, by using a competitive immunoassay kit (PGE_2 EIA Kit) from Enzo Life Science, according to the manufacturer's instructions. The values were related to protein content, as measured by the Bradford assay (Bio-Rad, USA).

3.6 Western-blot Analysis

As previously described [8], total, cytoplasmic and nuclear cellular protein extracts from HT-29 cells were prepared and analysed by Western-blotting. For total cellular protein extracts, washed cell pellets were resuspended in an ice-cold lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% (w/v) glycerol, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 1 mM PMSF, 1/100 (v/v) protease inhibitor cocktail) for 20 minutes, on ice. Cell debris was subsequently removed by centrifugation at 14000 rpm for 20 minutes at 4ºC and supernatants were then collected and stored at -20ºC. Cytoplasmic protein extracts were obtained essentially in the same way. Washed cells were lysed in an ice-cold buffer containing 10 mM Tris–HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 and 1% protease inhibitor cocktail, pH 7.5, for 5 minutes on ice. Afterwards, lysates were centrifuged at 5000 rpm for 5 minutes at 4ºC and the supernatants (cytoplasmic extracts) were collected and stored at -20ºC. The pellets were also collected and resuspended in an ice-cold buffer with 20 mM Hepes, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 300 mM NaCl, 20% (w/v) glycerol, and 1% protease inhibitor cocktail, pH 7.5 and left on ice for 30 minutes. Then, the mixture was centrifuged at 14000 rpm for 20 minutes at 4ºC and the supernatants (nuclear extracts) were saved at -80ºC.

Protein concentration was determined by using the Bio-Rad protein assay reagent (Bradford assay), according to the manufacturer's specifications (Bio-Rad, USA).

A range of 30-80 micrograms of reduced and denatured proteins were separated by SDS/PAGE electrophoresis on a 10% - 12% (v/v) acrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, UK) by electroblotting. To avoid non-specific binding, membranes were blocked with skimmed milk in TBS pH 7.6 supplemented with 0.1% (v/v) Tween 20 (TBS-T: 20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) and then probed with antibodies against iNOS, COX-2 and IκB-α,

overnight at 4ºC and against phospho-STAT1, phospho-p38 MAPK and phospho-SAPK/JNK 3 hours at room temperature, with a constant low shaking. After finishing, membranes were washed three times and further incubated with alkaline phosphatase-conjugated secondary antibodies (2 hours at room temperature and constant shaking). Immunoreactive bands were detected by fluorescence in a Typhoon 9000 scanner (Amersham Biosciences) and analysed with the ImageQuant TM software from Amersham Biosciences. After analysis of target proteins, each blot was stripped off and reprobed with the primary antibodies against β-actin, β-tubulin or lamin B1, used as controls for protein loading.

3.7 Total RNA Extraction and quantitative Real-Time RT-PCR (qRT-PCR)

Total RNA was extracted from HT-29 cells seeded in six-well-plates (8 x 10^5 cells/well), after 1 hour of pretreatment with Resv and/or 5-ASA followed by 6 hours of cytokine-challenge, using the RNA extraction kit Aurum ™ Total RNA Mini (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Extracted RNA was quantified using a NanoDrop ND-1000 spectrophotometer at 260 nm and its purity and integrity were assessed by ratio of absorbance at 240/260 and 280/260 nanometers. A constant amount of RNA (1 µg/sample) was reverse transcribed into cDNA, using the NZY First-Stand cDNA Synthesis Kit (NZYtech, Portugal), according to the manufacturer's protocol. PCR reactions were performed with 25 µg/ml of transcribed cDNA. The primers for iNOS, COX-2 and the housekeeping gene HPRT-1 (hypoxanthine phosphoribosyltransferase-1) were designed using the Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA) and the primers sequences were: iNOS, sense 5´- AATCCAGATAAGTGACATAAG -3', antisense 5'- CTCCACATTGTTGTTGAT -3'; COX-2, sense 5'- ATTATGAGTTTATGTGTTGAC -3'; antisense 5'- TAGGAGAGGTTAGAGAAG -3'; HPRT-1 sense 5'- TGACACTGGCAAAACAATG -3', antisense 5'- GGCTTATATCCAACACTTCG -3'. Real time-PCR was performed in 20 µl of total volume, containing 2 µl of each primer (250 nM), 2 µl of cDNA of each sample, 10 µl of the IQ™ SYBR Green Supermix (Bio-Rad) and RNase-free distilled water to make up the volume to 20 µl, in a CFX96 ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Thermal cycling conditions were the following: 3 minutes at 95 ºC to activate the iTaq™ DNA polymerase, then 45 cycles, each consisting of a denaturation step (95ºC, 10 seconds), an annealing step (55ºC, 30 seconds) and an elongation step (72ºC, 30 seconds). Fluorescence measures were taken every cycle at the

end of the annealing step and the specificity of the amplification products was evaluated through the analysis of the melting curve. The efficiency of the amplification reaction for each gene was calculated by running a standard curve of serially diluted cDNA sample. Gene expression was analysed using the Bio-Rad CFX Manager 3.0 software (Bio-Rad, Hercules, CA, USA), which enables the analysis of the results with the Pfaffl method. The results for each gene of interest were normalized against HPRT-1, the housekeeping gene found to be stable under experimental conditions and expressed as a percentage of control cells, *i.e.* cytokine-stimulated cells.

3.8 Fluorescence Confocal Microscopy

HT-29 colon epithelial cells were seeded onto glass coverslips on 24 well plates (1.5×10^5 cells/well) and treated with Resv and/or 5-ASA for 1 hour and then exposed to the cocktail of cytokines for 30 minutes. After this period, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Fixed cells were washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 2 minutes. After washing, cells were incubated overnight with phospho-STAT1 antibody diluted in PBS (1:50) at 4ºC. Then, the cells were washed twice with PBS, followed by incubation with Alexa Flour 594 conjugated secondary antibody diluted in PBS (1:100) for 4 hours at room temperature. After washing twice with PBS, the coverslips were mounted with glycerol and PBS containing the nucleic acid stain Hoechst (1 µg/ml). Cells were examined under a confocal microscope (Ziess LSM 510Meta).

3.9 Evaluation of Intracellular Reactive Species

Intracellular reactive species were assessed by using the non-fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), which permeates cell membranes and may be oxidized by reactive species, yielding the fluorescent 2′,7′-dichlorofluorescein (DCF) [34]. Briefly, cells in 12-well plates (4×10^5 cells/well) were previously incubated in the presence or in the absence of Resv and 5-ASA and further subjected to the combination of cytokines for 16 hours. After that period of time the cells were incubated with 5 µM $DCFH_2$-DA in DMSO, at 37°C, in the dark for 15 minutes. Cells were then washed with PBS and maintained in 0.5 ml of PBS during the fluorescence intensity measurements in a Synergy HT plate reader (Bio-Tek Instruments) (excitation and emission wavelengths at 485 and 530 nm, respectively).

Cells were also observed in an inverted fluorescence microscope (Zeiss Axiovert 40), using a FITC filter.

3.10 Statistical Analysis

All data were expressed as means ± SEM of at least 3 independent assays, each one in duplicate. Differences between groups were analysed by one-way analysis of variance (ANOVA) and Tukey's *post hoc* test was used as appropriate. Values of $p<0.05$ were accepted as statistically significant.

4. Results

4.1 Resv up to 25 µM did not affect HT-29 cells viability

The cytotoxicity of Resv alone or in combination with 5-ASA on HT-29 cell line was evaluated, upon 24 hours of cell incubation with the compounds, by the MTT assay. As illustrated in **Figure 2A**, neither Resv alone, in the concentration range of 12.5 to 25 µM, nor the combination of 25 µM Resv with 500 µM 5-ASA exerted cytotoxicity in HT-29 cells. However, at the concentration of 50 µM, Resv produced a small decrease in the cell viability relative to the control (untreated cells). Thus, concentrations of 25 µM Resv and 500 µM 5-ASA were chosen to perform the next experiments, since they proved to be subtoxic concentrations. As previously reported [8], the mixture of cytokines, at the concentrations selected as inflammatory stimulus in HT-29 cells, induced a decrease in cell viability to about 50 per cent (data not shown).

Considering that Resv in high doses may be hepatotoxic, we assessed the toxicity of Resv, 5-ASA and of both compounds in HepG2 cells as an *in vitro* model of human hepatocytes, in the same assay conditions. As illustrated in **Figure 2B**, none of the compounds induced loss of cell viability during this period of time.

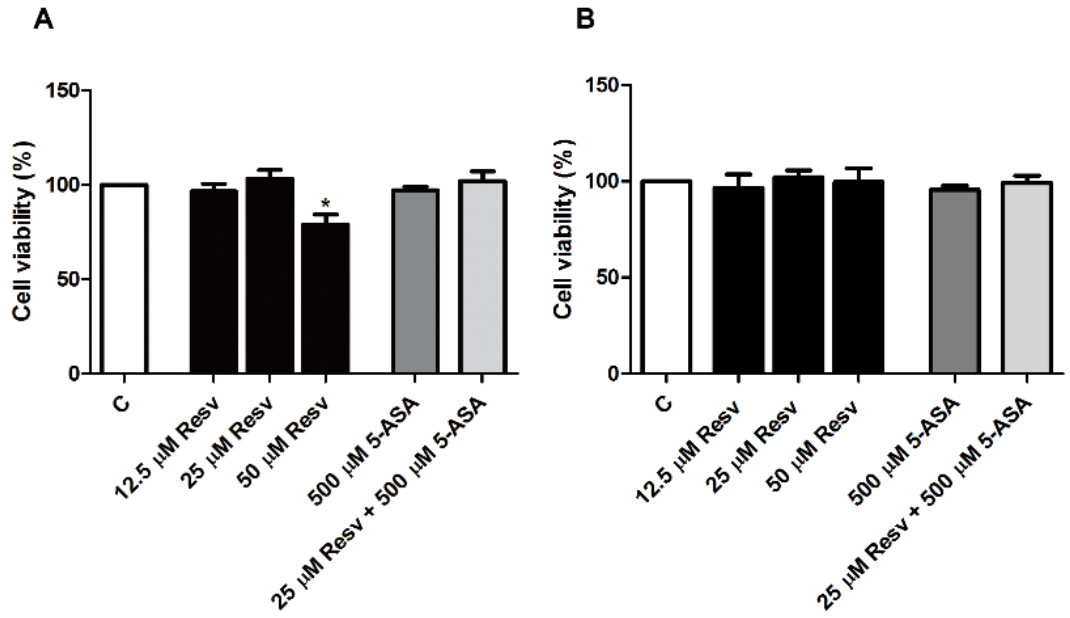


Figure 2 - Effects of Resv and/or 5-ASA on cell viability. Cells were incubated for 24 hours with different concentrations of Resv (12.5 to 50 µM), 5-ASA (500 µM) and a combination of 25 µM Resv and 500 µM 5-ASA. (A) HT-29 cell-line viability and (B) HepG2 cell-line viability were assessed by MTT reduction and determined as percentage of control cells (without compounds). Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{*}P<0.05$ vs Control.

4.2 Secretion of NO and PGE_2 was inhibited more efficiently by Resv than by 5-ASA, in stimulated HT-29 cells

To elucidate the ability of Resv alone or in combination with 5-ASA to inhibit the production of some pro-inflammatory mediators, the levels of NO and PGE_2 generated by cytokine-stimulated HT-29 cells were evaluated.

As shown in **Figure 3A**, stimulation of HT-29 cells with cytokines, for 24 hours, triggered a significant increase of cellular nitrite formation as compared to control (untreated cells), in agreement with previous studies. Cells treatment with 25 µM Resv, for 1 hour, before cytokine stimulation, significantly reduced the nitrite levels by about 50%. This inhibitory effect was not significantly different to that of 500 µM 5-ASA and occurred at a concentration 20 times lower. Also, the combination of 25 µM Resv with 500 µM 5-ASA did not cause any additional significant effect.

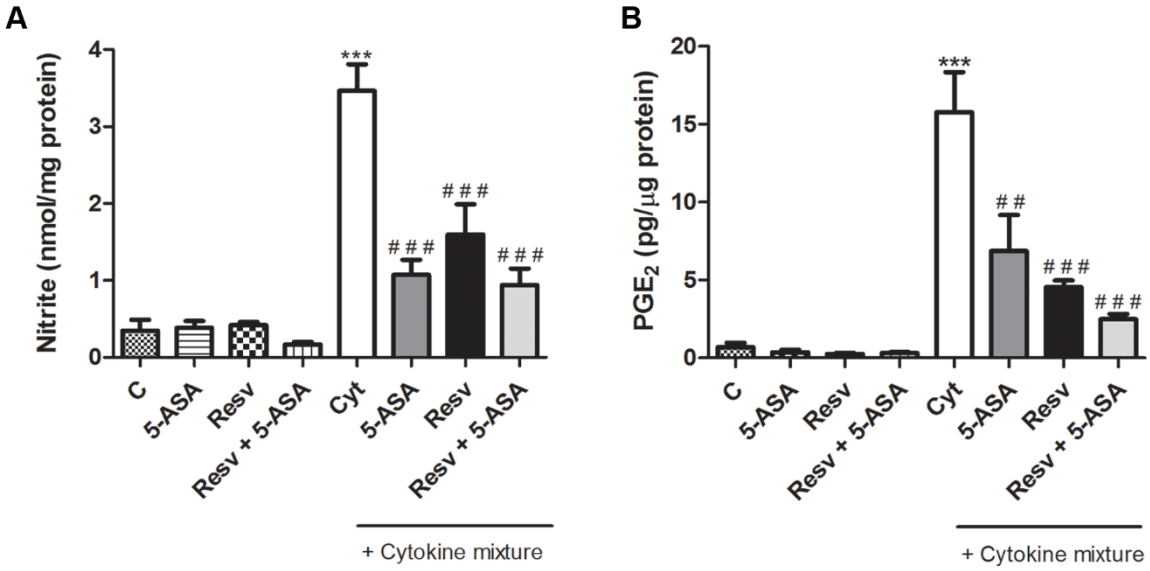


Figure 3 - Resv decreases cytokine–induced pro-inflammatory mediators production more efficiently than 5-ASA, in HT-29 cells. Cells were pre-incubated with 25 µM Resv or 500 µM 5-ASA or both (25 µM Resv plus 500 µM 5-ASA) and then exposed to cytokines (Cyt) for 24 hours or 16 hours for NO and PGE_2, respectively. The NO (A) and PGE_2 (B) production in cells was measured as described in "Materials and Methods". Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{***}P<0.001$ vs Control, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs Cytokines.

In order to draw comparison between Resv and 5-ASA on PGE_2 production, cells were treated with the compounds for 1 hour and then exposed to the cytokine mixture (IL-1α, TNF-α and IFN-γ) for 16 hours. In **Figure 3B**, it is patent that PGE_2 production was enhanced in response to cytokine treatment and that this increase was deeply inhibited by Resv by almost

75%, a higher inhibitory effect than that induced by 5-ASA (about 50%) at a concentration 20 times higher. The combined effect of Resv and 5-ASA seemed to be stronger than that of the individual compounds, however data are not statistically different.

4.3 Resv counteracted, in a greater extent than 5-ASA, cytokine-stimulated expression of iNOS and COX-2 proteins and mRNAs, in HT-29 cells

To further investigate whether the protection afforded by Resv, with regarding to the pro-inflammatory mediators studied, was related to the inhibition of the inducible forms of NO synthase and of cyclooxygenase, the protein expressions and mRNA levels were determined by Western blotting and qRT-PCR, respectively. Analysing **Figures 4** and **5**, it is clear that protein and mRNA levels of iNOS and COX-2, which were hardly detectable in non-stimulated cells, were significantly enhanced after cytokine exposure, according to data previously reported [8].

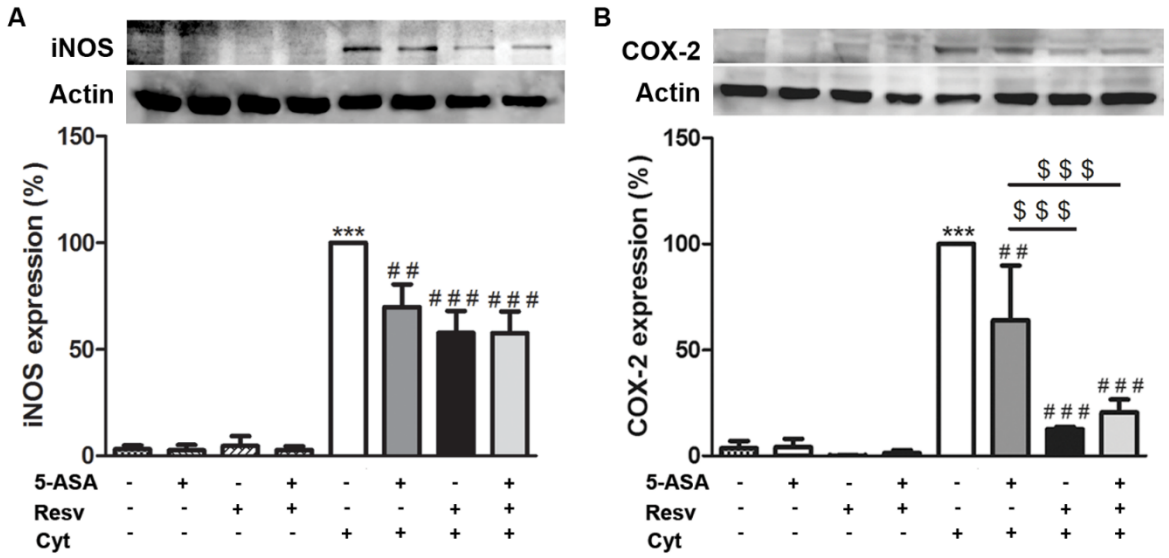


Figure 4 - Resv suppresses cytokine-induced iNOS and COX-2 expression more efficiently than 5-ASA, in HT-29 cells. Cells were pre-incubated with 25 µM Resv or 500 µM 5-ASA or both (25 µM Resv plus 500 µM 5-ASA) and then challenged with a combination of cytokines. iNOS (A) and COX-2 (B) expressions were evaluated after 24 hours or 16 hours, respectively, in total extracts by Western blotting, as described in "Materials and Methods", and expressed as percentage of cytokine-stimulated cells. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{***}P<0.001$ vs Control, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs Cytokines and $^{\$\$\$}P<0.001$ vs 5-ASA plus Cytokines.

However, cytokine stimulatory effect was significantly reduced by pre-treating the cells with Resv and/or 5-ASA, for 1 hour, before the exposure to cytokines. As illustrated in **Figures 4** and **5**, the extent to which Resv alone counteracted the cytokine-induced increase in COX-2 protein and mRNA levels was clearly and statistically higher than that assigned to 5-ASA, particularly if the different concentrations of the compounds are taken into account (25 µM Resv and 500 µM 5-ASA). However, the combination of Resv with 5-ASA did not promote an enhancement of this effect.

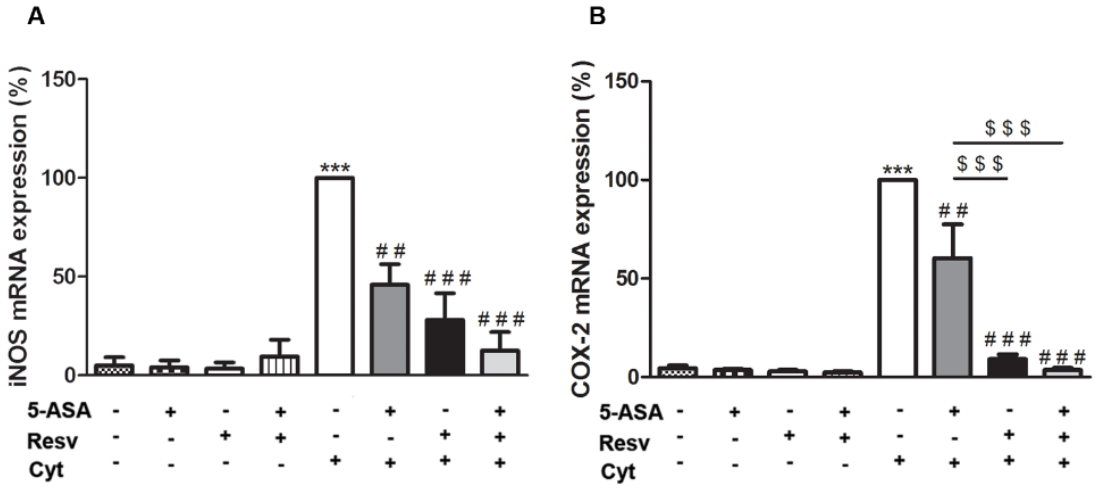


Figure 5 - Resv suppresses cytokine-induced iNOS and COX-2 mRNA levels more efficiently than 5-ASA, in HT-29 cells. Cells were pre-incubated with 25 µM Resv or 500 µM 5-ASA or both (25 µM Resv plus 500 µM 5-ASA) and then exposed to a combination of cytokines. iNOS (A) and COX-2 (B) mRNA production was evaluated after 6 hours by qRT-PCR, as described in "Materials and Methods", and expressed as percentage of cytokine-stimulated cells. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{***}P<0.001$ vs Control, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs Cytokines and $^{\$\$\$}P<0.001$ vs 5-ASA plus Cytokines.

4.4 Resv *per se* or in combination with 5-ASA did not prevent cytokine-induced IkB-α degradation, in HT-29 cells

In order to verify the involvement of NF-kB pathway in the protection afforded by Resv alone or in combination with 5-ASA, cells were stimulated with cytokines in the absence and presence of the compounds and the degradation of IkB-α was analysed by Western blotting. As previously observed [8], 30 minutes after cells stimulation, the mixture of cytokines induced the degradation of IkB-α, resulting in a decrease of this protein to about 75% relative to a control (non-treated cells). As shown in **Figure 6**, Resv was not able to prevent the degradation of IkB-α, either alone or associated with 5-ASA.

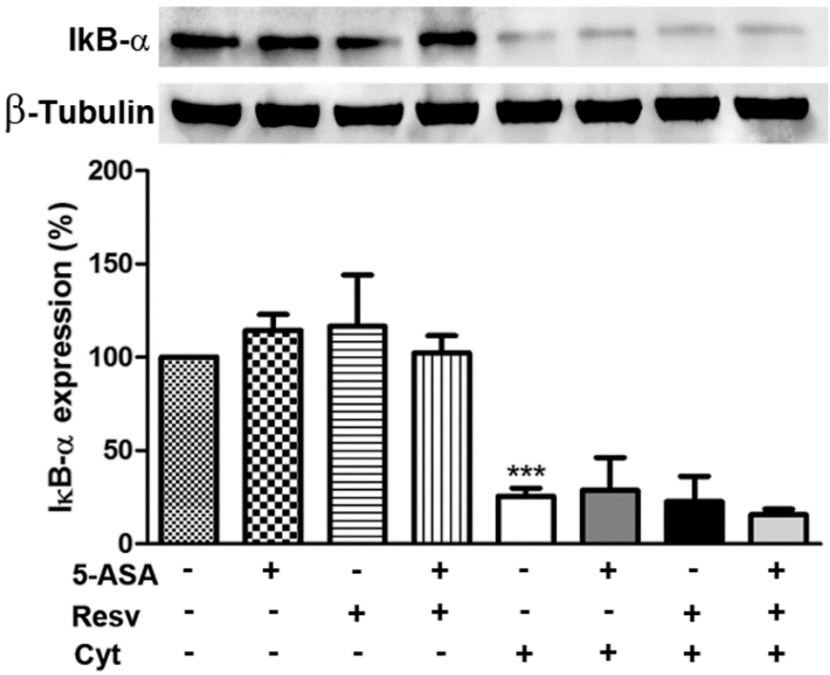


Figure 6 - Resv does not prevent IkB-α degradation induced by cytokines, in HT-29 cells. Cells were pre-incubated with 25 µM Resv or 500 µM 5-ASA or both (25 µM Resvplus 500 µM 5-ASA) and then exposed to a combination of cytokines for 30 minutes. IkB-α degradation was analysed in cytoplasmic extracts by Western blotting, as described in "Materials and Methods", and expressed as percentage of control cells, i.e. cells not treated. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{***}P<0.001$ vs Control.

4.5 Resv inhibited expression of pro-inflammatory markers in cytokine-stimulated HT-29 cells via JAK-STAT pathway

In pursuit of knowing more about the mechanisms underlying the protection afforded by Resv against inflammation, in our experimental conditions, further pathways beyond NF-kB were explored, namely that involving the transcription factor STAT1. Thus, the ability of Resv to decrease the levels of the tyrosine (Tyr) 701 phosphorylated form of this transcription factor in the nucleus was monitored. As verified in **Figure 7A**, Resv was able to inhibit the cytokine-induced levels of activated (Tyr701 phosphorylated) STAT1 in the nucleus in a similar way to 5-ASA, but at a concentration 20 times lower. The association of Resv with 5-ASA did not elicit an increase in efficiency in this pathway. In order to further illustrate the ability of the compounds under study in decreasing the amount of Tyr701 phospho-STAT1 in the nucleus of cytokine-stimulated HT-29 cells, immunocytochemical studies were performed. Representative confocal images of HT-29 cells shown in **Figure 7B** denoted nuclear staining patterns consistent with the results of immunoblotting of the nuclear extracts. Microscopic data obtained with 5-ASA plus cytokines and with the combination (Resv and 5-ASA) plus cytokines were very similar to those depicted in Figure 7B for Resv alone plus cytokines and, thus, they were not represented, for the sake of clarity.

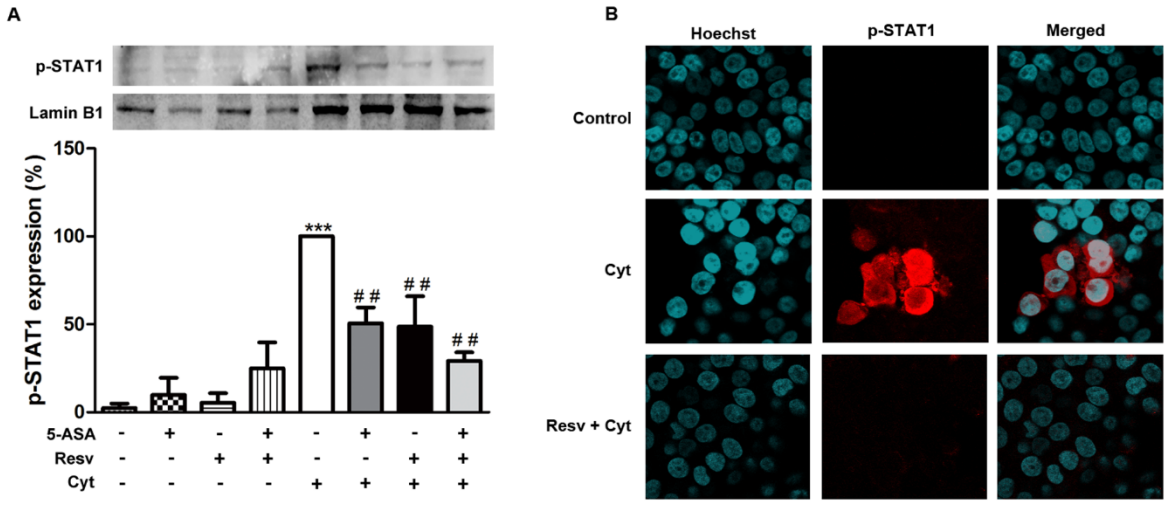


Figure 7 - Resv decreases activated-STAT1 levels in the nucleus of cytokine-stimulated HT-29 cells more efficiently than 5-ASA. Cells were pre-incubated with 25 µM Resv or 500 µM 5-ASA or both (25 µM Resv plus 500 µM 5-ASA) and then exposed to a combination of cytokines for 30 minutes. The levels of Tyr701 phospho-STAT1 were analysed in nuclear extracts by Western blotting (A), as described in "Materials and Methods" and expressed as percentage of cytokine-stimulated cells. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{***}P<0.001$ vs Control and $^{\#\#}P<0.01$ vs Cytokines. (B) Representative confocal microscopy pictures of non-stimulated, cytokine-stimulated and Resv pre-incubated HT-29 cells. Simultaneous DNA labelling with Hoechst was performed to visualize the nuclear compartments.

4.6 The protection afforded by Resv alone or in combination with 5-ASA against cytokine-induced inflammation involved MAPKs signalling, in HT-29 cells

Being aware that the upstream kinases, such as p38 and SAPK/JNK MAPKs, play an important role in the regulation of the activation of several transcription factors, we also evaluated the effects of Resv, alone or in combination with 5-ASA, on p38 MAPK and SAPK/JNK phosphorylation (activation). It was observed (**Figure 8A** and **8B**) that the combination of cytokines, after 30 min of cell-stimulation, induced the phosphorylation of p38 MAPK and of SAPK/JNK. Resv alone or in combination with 5-ASA maintained the levels of activated p38 MAPK in HT-29 cells (**Figure 8A**), but counteracted the cytokine-induced activation of SAPK/JNK, in those cells (**Figure 8B**). On the other hand, 5-ASA alone, in such activated cells, did not affect neither p38 MAPK nor SAPK/JNK activation. However, cells pre-incubation with 5-ASA plus Resv significantly increased the suppressive effect of Resv on SAPK/JNK phosphorylation.

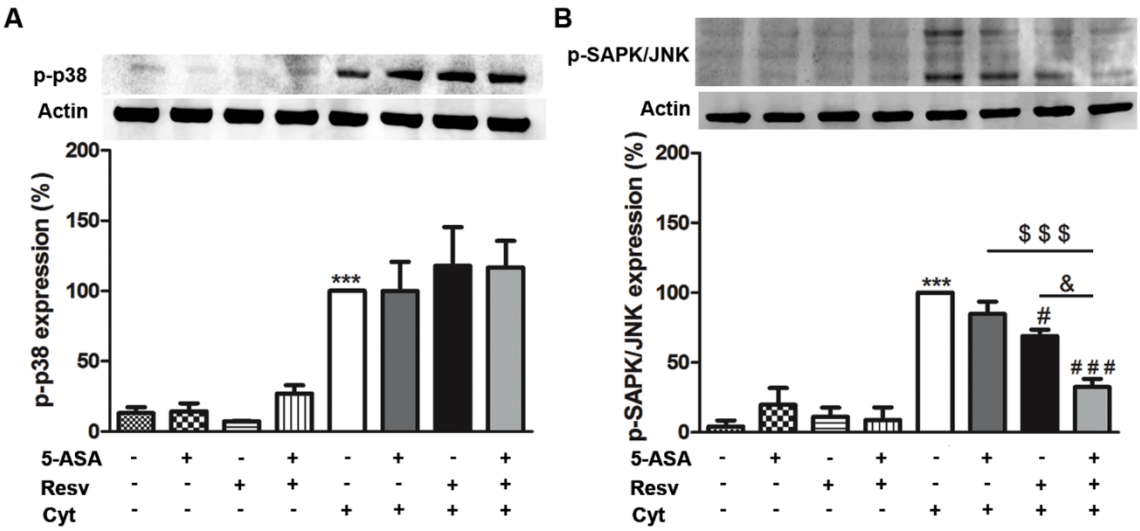


Figure 8 - p38 MAPK and SAPK/JNK are involved in resveratrol protection, alone or plus 5-ASA, in cytokine-stimulated HT-29 cells. Cells were pre-incubated with 25 µM Resv or 500 µM 5-ASA or both (25 µM Resv plus 500 µM 5-ASA) and then exposed to a combination of cytokines for 30 minutes. Phospho-p38 MAPK (A) and phospho-SAPK/JNK (B) expressions were evaluated in total extracts by Western blotting, as described in "Materials and Methods", and expressed as percentage of cytokine-stimulated cells. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{***}P<0.001$ vs Control (non-treated cells), $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs Cytokines, $^{\$\$\$}P<0.001$ vs 5-ASA plus Cytokines and $^{\&}P<0.05$ vs Resv plus Cytokines.

4.7 Resv, unlike 5-ASA, exerted a strong inhibition in the generation of intracellular reactive species, in cytokine-stimulated HT-29 cells

It is well known that the formation of intracellular reactive species is close related to the inflammatory process. Thus, the ability of Resv, in comparison and associated with 5-ASA, to inhibit cytokine-induced intracellular oxidative stress was assessed by dichlorodihydrofluorescein fluorescence. A time-course analysis was carried out, following cytokine challenge. As shown on the top of **Figure 9**, the intracellular levels of reactive species started to increase after 8 hours of cell incubation with cytokines and were maintained until 24 hours. To assess the effects of Resv and/or 5-ASA, the experiment was conducted with 1 hour of cell pre-incubation with the compounds, followed by 16 hours of incubation with cytokines. As evidenced in the bar graph and typically shown in the pictures on the right of **Figure 9**, Resv exhibited a stronger efficiency as an antioxidant than 5-ASA, and the association Resv and 5-ASA seems not to potentiate the effect of Resv *per se*.

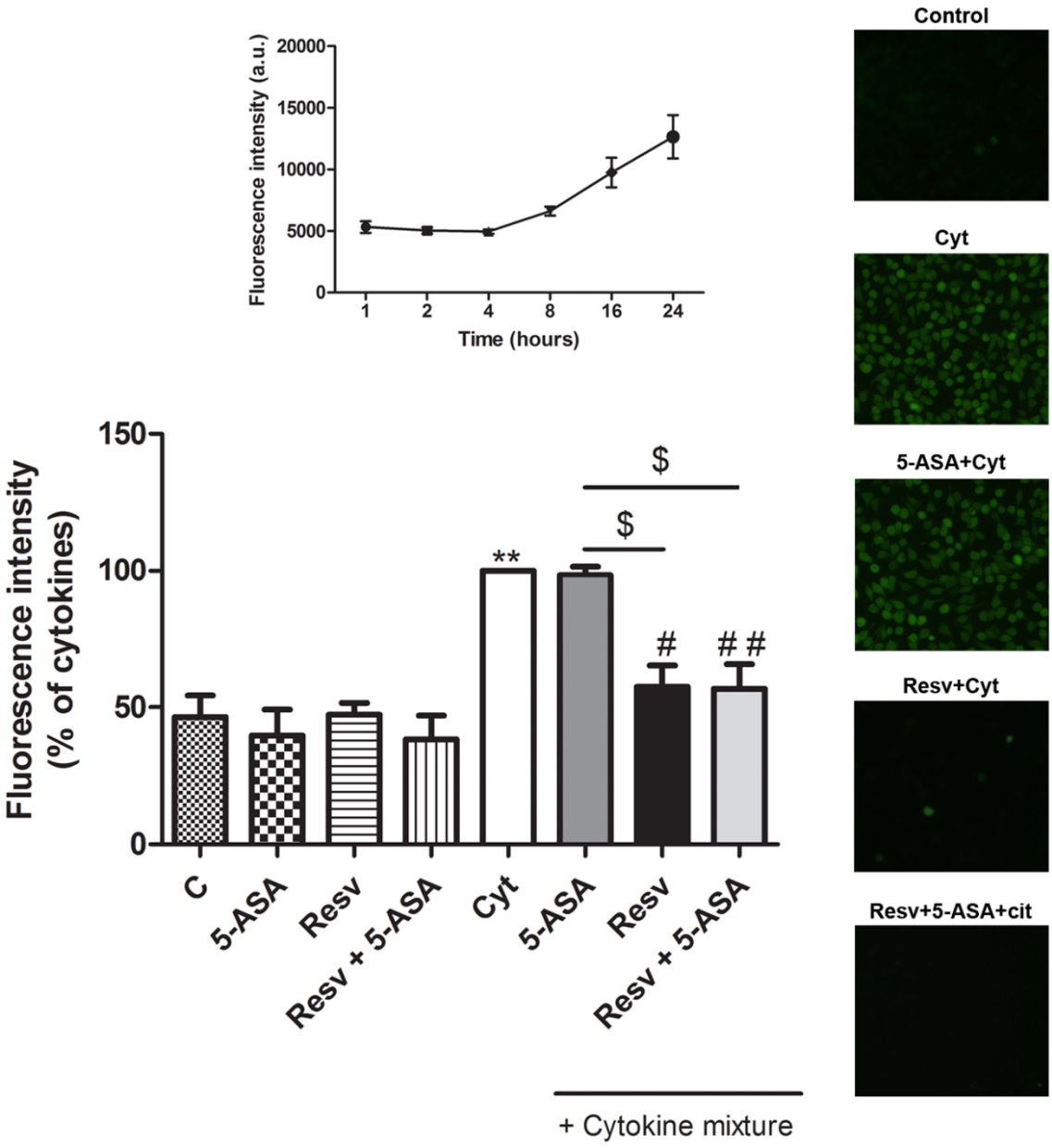


Figure 9 - Resv inhibits cytokine-induced oxidative stress in a greater extent than 5-ASA, in HT-29 cells. Cells were pre-incubated with 25 µM Resv or 500 µM 5-ASA or both (25 µM Resv plus 500 µM 5-ASA) for 1 hour and then exposed to a combination of cytokines for 16 hours. Reactive species production was measured after 16 hours of incubation with cytokines, by oxidation of the probe dichlorodihydrofluorescein, expressed in terms of fluorescence intensity relative to cytokine-stimulated HT-29 cells. A time-course of HT-29 reactive species production following cytokine challenge is presented on the top and representative images obtained by fluorescence microscopy (400x) of cells at 16 hours after cytokine treatment, in the absence or presence of 25 µM Resv and/or 500 µM 5-ASA are presented on the right. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{**}P<0.01$ vs Control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs Cytokines and $^{\$}P<0.05$ vs 5-ASA plus Cytokines.

5. Discussion

The growing knowledge on the cell signalling pathways, underlying the inflammatory process that characterizes Inflammatory Bowel Disease [22, 35], has allowed the investigation of better target strategies to limit IBD progression. However, despite the advances in the pharmacological treatment that have been made in the last years, this disease remains devoid of cure and, consequently, the pharmacological management is mainly used to prevent and to treat symptoms and still induce or maintain the remission periods [23]. On the other hand, given that IBD patients often become refractory to the existing therapies, several lines of evidence show that many patients take some form of dietary supplement to achieve extra benefits [23, 36].

The anti-inflammatory role of polyphenols in chronic inflammatory diseases as IBD has been supported by many authors, who believe that the consumption of these biological phytochemicals can be highly advantageous to prevent or limit disease progression [5, 6, 8]. Resveratrol is a non-flavonoid polyphenol, particularly abundant in grapes and red-wine, whose action mechanisms have been extensively studied in the last decades. However, one of the major concerns about Resv efficacy is related to its low oral bioavailability [16, 17]. In fact, it is estimated that after oral intake, Resv can be extensively metabolized in the liver and intestine, resulting in a very low bioavailability in humans [10, 16]. Nevertheless, there are evidences demonstrating that this compound is able to accumulate in specific tissues, particularly in the intestinal tissue, where its glucuronic acid and sulfate conjugates, the major metabolites of Resv, may work as a pool of the active compound that is released upon the action of β-glucuronidases or sulfatases [16, 37]. This suggests that Resv may be particularly attractive to modulate the inflammatory process settled in IBD patients.

In the present study, it was first examined the impact of Resv, alone or in combination with the pharmaceutical agent 5-ASA, on the HT-29 cell viability. Afterward, considering that beyond its bioavailability, another major concern about Resv helpfulness is related to some studies pointing out that the consumption of high doses of polyphenols may be deleterious to the liver [18, 38], the possible hepatotoxicity of our compounds was assessed on HepG2 cell line. To exclude any possible toxicity, the selected concentrations for the next experiments were 25 µM Resv and 500 µM 5-ASA. The reported capacity of Resv to reduce the levels of some pro-inflammatory mediators in macrophages [13] motivated us to verify its ability to counteract the induction of NO and PGE_2 production by cytokines in HT-29 cells, comparing its action

with that of the drug 5-ASA. Resv showed to be efficient in inhibiting cytokine-induced NO and PGE_2 production, in a concentration 20 times lower than 5-ASA. Accordingly, in a study by Zhong *et al* [12], Resv showed similar effects in LPS-stimulated BV-2 microglial cells. The combination of Resv and 5-ASA did not give any evidence of enhanced efficiency, in our experimental conditions.

To assess whether the above protective effect of Resv, concerning pro-inflammatory mediators, was exerted via inhibition of iNOS and COX-2, both protein and mRNA production were evaluated. Our results showed that Resv can suppress transcriptionally the cytokine-induction of these two enzymes, in a greater extent than 5-ASA, revealing its anti-inflammatory superiority and, on the other hand, that the inhibition of iNOS and COX-2 are related to the reduction of NO and PGE_2 production induced by Resv. This is consistent with the results previously reported by Cianciulli *et al* [39], showing the ability of Resv to inhibit LPS-induced COX-2 and PGE_2 production, in Caco-2 cells.

The transcriptional regulation of pro-inflammatory markers is a strictly controlled event regulated by several transcriptional factors as NF-kB. NF-kB pathway is one of the most studied regulators of the transcription of pro-inflammatory genes, such as those of iNOS and COX-2. Besides, it is known that this pathway is usually induced in the intestine of IBD patients [29]. It turns out that in our experimental conditions, Resv could not prevent the degradation of IkB-α which is a well-established step for the classical activation of NF-kB pathway [29]. Our results are, hence, apparently in contradiction to those reported by Zhong *et al* [12], demonstrating the inhibition of LPS-induced activation of NF-kB pathway by Resv in microglial cells. In fact, in our study, Resv showed to be not able to down-regulate the activation of NF-kB pathway, either alone or in combination with 5-ASA. Therefore, the anti-inflammatory effect of Resv should be explained by the involvement of an alternative cell signalling pathway, such as JAK-STAT pathway, which is also induced in IBD patients [32]. In a previous study from our group, it was demonstrated for the first time, the ability of 5-ASA to decrease the amount of activated STAT1 in the nucleus of HT-29 cells, by evaluating the levels of phosphorylated STAT1 at Tyr701 [8]. It is believed that after IFN-γ-receptor stimulation, STAT1 is phosphorylated at Tyr701, being this event essential for STAT1 dimerization, its translocation to the nucleus and DNA binding or, in other words, for STAT1 activation [40]. In the present study, Resv ability to reduce the levels of Tyr701 phosphorylated STAT1 in the nucleus of HT-29 cells was clearly demonstrated. The combination of Resv with 5-ASA showed a similar effect to that of the compounds alone. To our knowledge, no study dealing with the ability of Resv to reduce

cytokine-increased levels of Tyr701 phosphorylated STAT1, in the nucleus of HT-29 cells, has been reported yet. Moreover, this finding is in line with the observation of Capiralla *et al* [41], demonstrating the suppressive effect of Resv on LPS-induced phosphorylation of STAT1 at Tyr701 in RAW 264.7 macrophages and BV-2 microglial cells.

The observed reduction of activated STAT1 accumulated in the nucleus induced by Resv in HT-29 cells, may have an extremely important impact in the context of IBD, since besides its implications for the inhibition of many pro-inflammatory genes, it can contribute for the prevention of radiation resistance acquired by IBD patients during radiotherapy treatment, which has been associated to high levels of nuclear STAT1 [42]. In fact, IBD patients exhibit a high risk of developing colorectal cancer as compared to healthy population [43] and, for this reason, the use of anti-inflammatory compounds with the potential to inhibit that event (acting as anticancer agents) can be of great importance in IBD context. Therefore, taking into account the range of concentrations at which Resv and 5-ASA exert anti-inflammatory effects, Resv proved to give further benefits in the context of IBD as compared to 5-ASA.

Seeking more information about the protective role of Resv as compared to 5-ASA, the involvement of MAPK pathway was studied. MAPKs are a group of enzymes considered as instigative controllers of many downstream signalling pathways, with relevance, for example, in the activation of some transcription factors [31]. The most known subfamilies of MAPKs are ERK 1/2, SAPK/JNK and p38 MAPK. ERK 1/2 is strongly activated by growth factors and in a lesser extent by cytokines [44]. In contrast, SAPK/JNK and p38 MAPK are strongly activated by cytokines, such as TNF-α [44]. This report shows that, contrarily to what happens with 5-ASA, Resv inhibited cytokine-induced phospho-SAPK/JNK levels, in HT-29 cells. The combination of Resv with 5-ASA seemed to enhance the ability of Resv to decrease the levels of this phosphorylated protein. Large body of evidence suggests that SAPK/JNK pathway is an important signal transduction pathway implicated in IBD [45] and for this reason there is a recent considerable interest in the development of anti-JNK therapies [45]. Towards this goal, several studies have been conducted demonstrating the effectiveness of some JNK inhibitors in the protection against pathophysiology features of experimentally induced IBD in some *in vivo* models [46, 47]. On the other hand, SAPK/JNK pathway is also involved in the phosphorylation of STAT1 at Serine 727 [48] and it is known that this specific phosphorylation is required for the maximization of the transcriptional potential of STAT1. In fact this post-translational modification is important, for instance, for the modulation of the interaction of STAT1 with co-activator proteins [49]. Thus, our present data suggest that the anti-inflammatory protection

afforded by either Resv alone or in combination with 5-ASA involves the prevention of SAPK/JNK activation and the subsequent impairment of the maximization of STAT1 transcriptional potential. This is a meaningful finding in the light of the therapeutic potential of JNK inhibitors.

Besides, there is accumulating evidence that p38 MAPK can also mediate the STAT1 Ser727 phosphorylation [48, 50]. This event would enhance PIAS1 (protein inhibitor of activated STAT1) binding and SUMO-1 (small ubiquitin-related modifier-1) conjugation to STAT1 [51]. PIAS are a family of proteins implicated in the inhibition of STAT-mediated gene activation through many mechanisms, such as inhibiting DNA binding and promoting SUMO conjugation of STAT1 [40]. SUMOylation is a post-transcriptional modification that, in the case of STAT1, seems to function as a negative regulator, since inhibits STAT1 Tyr701 phosphorylation, prevents STAT1 DNA binding and also promotes its dephosphorylation [40, 51-53]. Thus, p38 MAPK induced maximization of STAT1 transcriptional potential (by phosphorylation on Ser727) and this event would precede the relatively slow emergence of STAT1 SUMOylation, triggering a negative feedback loop through PIAS1 and SUMO recruitment [52]. Remarkably, our data show that cytokine-induced activation (phosphorylation) of p38 MAPK was not counteracted by Resv and/or 5-ASA. This probably means that the protective effect of Resv or Resv plus 5-ASA takes advantage of the p38 MAPK-mediated negative feedback of STAT1. These findings strengthen our knowledge regarding the pathways involved in the protection afforded by Resv, 5-ASA and the combination of Resv with 5-ASA, in activated intestinal cells.

On the other hand, several studies demonstrate that oxidative stress and inflammation are closely related and therefore persistently elevated levels of ROS can contribute to the perpetuation of the inflammatory process and ultimately to cancer [54]. For this reason, we evaluated the effect of our compounds in the production of ROS. Interestingly, Resv pre-treatment of HT-29, before cytokine challenging, prevented the cytokine-induction of oxidative stress in these cells, which was not verified with 5-ASA pre-treatment. Thus, it is noteworthy that Resv has a more efficient antioxidant activity than 5-ASA in this type of cells.

In conclusion, under our experimental conditions, Resv revealed a stronger anti-inflammatory and antioxidant activity than 5-ASA, given that in a concentration 20 times lower, Resv was able to efficiently decrease cytokine-induced pro-inflammatory mediators (NO and PGE_2) production, pro-inflammatory enzymes (iNOS and COX-2) expression and intracellular reactive species formation. Moreover, in spite of not being able to prevent cytokine-induced IkB-α degradation, Resv efficiently decreased the amount of Tyr701

phosphorylated STAT1 in the nucleus of HT-29 cells, suggesting that JAK-STAT pathway is one of the key cascades involved in its anti-inflammatory activity. On the other hand, in contrast to 5-ASA, Resv was also able to inhibit the activation of the SAPK/JNK pathway, preventing the transcriptional potential maximization of the remaining Tyr701-phosphorylated STAT1. Furthermore Resv takes advantage of the negative feedback of STAT1, thought p38 MAPK pathway.

Overall, Resv did not exhibit a synergistic effect with 5-ASA.

Put together, data collected from our previous work and gathered from the present work support our belief that two polyphenols with completely different chemical structures, cyanidin-3-glucoside and resveratrol, abundant in a Mediterranean Diet, can assume a more efficient anti-inflammatory role than that of 5-ASA, a well-known pharmacological agent, used as the cornerstone of treatment for IBD patients. However, we are aware that the therapeutic value of these compounds must be confirmed by *in vivo* experiments, which are planned in a near future.

Considering that current treatment options for IBD patients are not completely successful, the Mediterranean Diet (rich in those polyphenolic compounds) can be envisaged as an interesting strategy to promote remission periods in IBD patients, limiting IBD progression and even to obviate colorectal cancer, which is commonly inflicted on these patients.

6. References

1. Bahadoran, Z., Mirmiran, P. & Azizi, F. Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. *J Diabetes Metab Disord* **12**, 43 (2013).
2. Vauzour, D., Rodriguez-Mateos, A., Corona, G., Oruna-Concha, M.J. & Spencer, J.P. Polyphenols and human health: prevention of disease and mechanisms of action. *Nutrients* **2**, 1106-31 (2010).
3. Scalbert, A., Manach, C., Morand, C., Remesy, C. & Jimenez, L. Dietary polyphenols and the prevention of diseases. *Crit Rev Food Sci Nutr* **45**, 287-306 (2005).
4. Manach, C., Scalbert, A., Morand, C., Remesy, C. & Jimenez, L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**, 727-47 (2004).
5. Romier, B., Schneider, Y.J., Larondelle, Y. & During, A. Dietary polyphenols can modulate the intestinal inflammatory response. *Nutr Rev* **67**, 363-78 (2009).
6. Martin, A.R., Villegas, I., Sanchez-Hidalgo, M. & de la Lastra, C.A. The effects of resveratrol, a phytoalexin derived from red wines, on chronic inflammation induced in an experimentally induced colitis model. *Br J Pharmacol* **147**, 873-85 (2006).
7. Paixao, J., Dinis, T.C. & Almeida, L.M. Malvidin-3-glucoside protects endothelial cells up-regulating endothelial NO synthase and inhibiting peroxynitrite-induced NF-kB activation. *Chem Biol Interact* **199**, 192-200 (2012).
8. Serra, D., Paixao, J., Nunes, C., Dinis, T.C. & Almeida, L.M. Cyanidin-3-glucoside suppresses cytokine-induced inflammatory response in human intestinal cells: comparison with 5-aminosalicylic acid. *PLoS One* **8**, e73001 (2013).
9. Paixao, J., Dinis, T.C. & Almeida, L.M. Dietary anthocyanins protect endothelial cells against peroxynitrite-induced mitochondrial apoptosis pathway and Bax nuclear translocation: an in vitro approach. *Apoptosis* **16**, 976-89 (2011).
10. Udenigwe, C.C., Ramprasath, V.R., Aluko, R.E. & Jones, P.J. Potential of resveratrol in anticancer and anti-inflammatory therapy. *Nutr Rev* **66**, 445-54 (2008).
11. Lippi, G., Franchini, M., Favaloro, E.J. & Targher, G. Moderate red wine consumption and cardiovascular disease risk: beyond the "French paradox". *Semin Thromb Hemost* **36**, 59-70 (2010).
12. Zhong, L.M. et al. Resveratrol inhibits inflammatory responses via the mammalian target of rapamycin signalling pathway in cultured LPS-stimulated microglial cells. *PLoS One* **7**, e32195 (2012).

13. Zong, Y. et al. Resveratrol inhibits LPS-induced MAPKs activation via activation of the phosphatidylinositol 3-kinase pathway in murine RAW 264.7 macrophage cells. *PLoS One* **7**, e44107 (2012).
14. Larrosa, M. et al. Effect of a low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS-induced colitis rat model. *J Agric Food Chem* **57**, 2211-20 (2009).
15. Bereswill, S. et al. Anti-inflammatory effects of resveratrol, curcumin and simvastatin in acute small intestinal inflammation. *PLoS One* **5**, e15099 (2010).
16. Walle, T. Bioavailability of resveratrol. *Ann N Y Acad Sci* **1215**, 9-15 (2011).
17. Cottart, C.H., Nivet-Antoine, V., Laguillier-Morizot, C. & Beaudeux, J.L. Resveratrol bioavailability and toxicity in humans. *Mol Nutr Food Res* **54**, 7-16 (2010).
18. Hassan-Khabbar, S. et al. Postischemic treatment by trans-resveratrol in rat liver ischemia-reperfusion: a possible strategy in liver surgery. *Liver Transpl* **14**, 451-9 (2008).
19. Larrosa, M. et al. Preventive oral treatment with resveratrol pro-prodrugs drastically reduce colon inflammation in rodents. *J Med Chem* **53**, 7365-76 (2010).
20. Rahal, K. et al. Resveratrol has antiinflammatory and antifibrotic effects in the peptidoglycan-polysaccharide rat model of Crohn's disease. *Inflamm Bowel Dis* **18**, 613-23 (2012).
21. Maloy, K.J. & Powrie, F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* **474**, 298-306 (2011).
22. Xavier, R.J. & Podolsky, D.K. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **448**, 427-34 (2007).
23. Triantafillidis, J.K., Merikas, E. & Georgopoulos, F. Current and emerging drugs for the treatment of inflammatory bowel disease. *Drug Des Devel Ther* **5**, 185-210 (2011).
24. Feghali, C.A. & Wright, T.M. Cytokines in acute and chronic inflammation. *Front Biosci* **2**, d12-26 (1997).
25. Beck, P.L. & Wallace, J.L. Cytokines in inflammatory bowel disease. *Mediators Inflamm* **6**, 95-103 (1997).
26. Kolios, G., Brown, Z., Robson, R.L., Robertson, D.A. & Westwick, J. Inducible nitric oxide synthase activity and expression in a human colonic epithelial cell line, HT-29. *Br J Pharmacol* **116**, 2866-72 (1995).
27. Kolios, G. et al. Ciprofloxacin inhibits cytokine-induced nitric oxide production in

human colonic epithelium. *Eur J Clin Invest* **36**, 720-9 (2006).

28. Paul, S. et al. Anti-inflammatory action of pterostilbene is mediated through the p38 mitogen-activated protein kinase pathway in colon cancer cells. *Cancer Prev Res (Phila)* **2**, 650-7 (2009).
29. Atreya, I., Atreya, R. & Neurath, M.F. NF-kappa B in inflammatory bowel disease. *J Intern Med* **263**, 591-596 (2008).
30. Feng, Y.J. & Li, Y.Y. The role of p38 mitogen-activated protein kinase in the pathogenesis of inflammatory bowel disease. *J Dig Dis* **12**, 327-32 (2011).
31. Broom, O.J., Widjaya, B., Troelsen, J., Olsen, J. & Nielsen, O.H. Mitogen activated protein kinases: a role in inflammatory bowel disease? *Clin Exp Immunol* **158**, 272-80 (2009).
32. Schreiber, S. et al. Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. *Gut* **51**, 379-85 (2002).
33. Wright, K., Ward, S.G., Kolios, G. & Westwick, J. Activation of phosphatidylinositol 3-kinase by interleukin-13. An inhibitory signal for inducible nitric-oxide synthase expression in epithelial cell line HT-29. *J Biol Chem* **272**, 12626-33 (1997).
34. Halliwell, B. & Whiteman, M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol* **142**, 231-55 (2004).
35. Kaser, A., Zeissig, S. & Blumberg, R.S. Inflammatory bowel disease. *Annu Rev Immunol* **28**, 573-621 (2010).
36. Frontela, C., Canali, R. & Virgili, F. [Use of dietary phenols to modulate inflammatory bowel response]. *Gastroenterol Hepatol* **33**, 307-12 (2010).
37. Kuhnle, G. et al. Resveratrol is absorbed in the small intestine as resveratrol glucuronide. *Biochem Biophys Res Commun* **272**, 212-7 (2000).
38. Martin, K.R. Polyphenols as dietary supplements: A double-edged sword. *Dovepress* **2**, 1-12 (2010).
39. Cianciulli, A. et al. Modulation of NF-κB activation by resveratrol in LPS treated human intestinal cells results in downregulation of PGE2 production and COX-2 expression. *Toxicology in Vitro* **26**, 1122-1128 (2012).
40. Shuai, K. & Liu, B. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol* **3**, 900-11 (2003).
41. Capiralla, H. et al. Resveratrol mitigates lipopolysaccharide- and Abeta-mediated

microglial inflammation by inhibiting the TLR4/NF-kappaB/STAT signalling cascade. *J Neurochem* **120**, 461-72 (2012).

42. Nardozzi, J.D., Lott, K. & Cingolani, G. Phosphorylation meets nuclear import: a review. *Cell Commun Signal* **8**, 32 (2010).
43. Williams, C., Panaccione, R., Ghosh, S. & Rioux, K. Optimizing clinical use of mesalazine (5-aminosalicylic acid) in inflammatory bowel disease. *Therap Adv Gastroenterol* **4**, 237-48 (2011).
44. Roux, P.P. & Blenis, J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* **68**, 320-44 (2004).
45. Roy, P.K., Rashid, F., Bragg, J. & Ibdah, J.A. Role of the JNK signal transduction pathway in inflammatory bowel disease. *World J Gastroenterol* **14**, 200-2 (2008).
46. Kersting, S. et al. The impact of JNK inhibitor D-JNKI-1 in a murine model of chronic colitis induced by dextran sulfate sodium. *J Inflamm Res* **6**, 71-81 (2013).
47. Reinecke, K. et al. The JNK inhibitor XG-102 protects against TNBS-induced colitis. *PLoS One* **7**, e30985 (2012).
48. Zhang, Y., Cho, Y.Y., Petersen, B.L., Zhu, F. & Dong, Z. Evidence of STAT1 phosphorylation modulated by MAPKs, MEK1 and MSK1. *Carcinogenesis* **25**, 1165-75 (2004).
49. Varinou, L. et al. Phosphorylation of the Stat1 transactivation domain is required for full-fledged IFN-gamma-dependent innate immunity. *Immunity* **19**, 793-802 (2003).
50. Goh, K.C., Haque, S.J. & Williams, B.R. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. *EMBO J* **18**, 5601-8 (1999).
51. Vanhatupa, S., Ungureanu, D., Paakkunainen, M. & Silvennoinen, O. MAPK-induced Ser727 phosphorylation promotes SUMOylation of STAT1. *Biochem J* **409**, 179-85 (2008).
52. Gronholm, J. et al. Structure-function analysis indicates that sumoylation modulates DNA-binding activity of STAT1. *BMC Biochem* **13**, 20 (2012).
53. Droescher, M., Begitt, A., Marg, A., Zacharias, M. & Vinkemeier, U. Cytokine-induced paracrystals prolong the activity of signal transducers and activators of transcription (STAT) and provide a model for the regulation of protein solubility by small ubiquitin-like modifier (SUMO). *J Biol Chem* **286**, 18731-46 (2011).

54. Surh, Y.J., Kundu, J.K., Na, H.K. & Lee, J.S. Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. *J Nutr* **135**, 2993S-3001S (2005).

Chapter 4

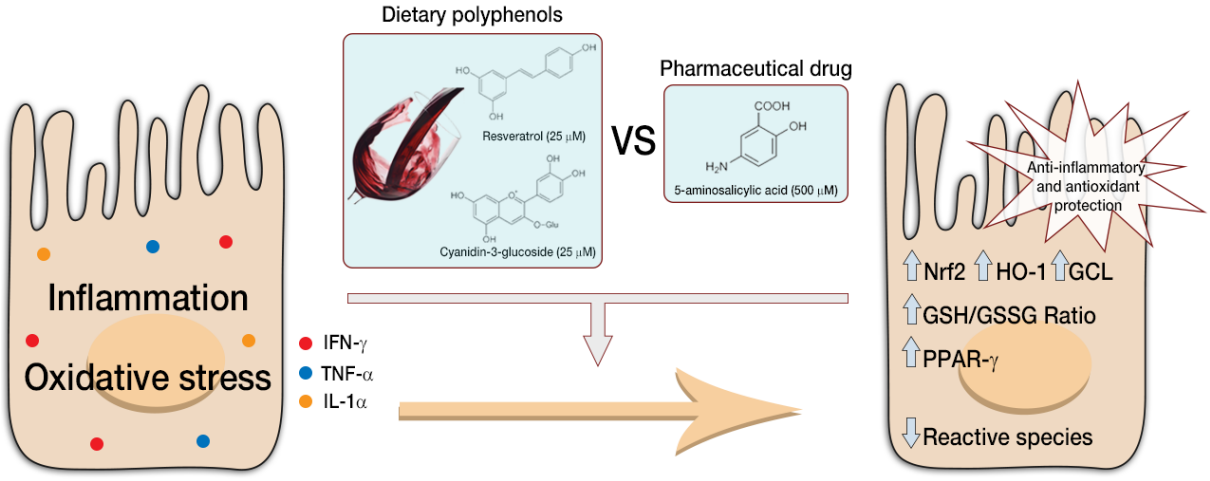
Role of Nrf2 and PPAR-γ in the protection afforded by cyanidin-3-glucoside and resveratrol against inflammation and oxidative stress in human intestinal cells: comparison with 5-aminosalicylic acid

Serra D, Almeida LM and Dinis TCP (**2015**), *"Role of Nrf2 and PPAR-γ in the protection afforded by cyanidin-3-glucoside and resveratrol against inflammation and oxidative stress in human intestinal cells: comparison with 5-aminosalicylic acid", submitted to Journal of Nutritional Biochemistry.*

1. Abstract

Dietary polyphenols, allying their valuable anti-inflammatory activity to their antioxidant effect, have been envisaged as promising adjuvant approaches to conventional drugs usually prescribed for patients with Inflammatory Bowel Disease (IBD). This study investigated whether the nuclear factor erythroid 2 (Nrf2) and peroxisome proliferator-activated receptor-gamma (PPAR-γ) pathways were involved in the protection afforded by two polyphenols highly abundant in red-wine, cyanidin-3-glucoside and resveratrol, against cytokine-induced inflammation and oxidative insult in HT-29 cells, in comparison with the drug, 5-aminosalicylic acid (5-ASA). Our data show that cyanidin-3-glucoside and resveratrol induced Nrf2 activation, increased hemoxygenase-1 and glutamate cysteine ligase mRNA expression, enhanced reduced glutathione to oxidized glutathione ratio and inhibited reactive species production, in cytokine-challenged cells, at much lower concentrations than 5-ASA. Unlike cyanidin-3-glucoside, resveratrol and 5-ASA also increased nuclear levels of PPAR-γ in cytokine-stimulated cells. In conclusion, both polyphenols might be interesting as nutraceuticals for the prevention of IBD progression.

2. Graphical Abstract



Representative scheme of some signalling pathways involved in the protection afforded by C3G and Resv against cytokine-induced inflammation and oxidative response in HT-29 cells, in comparison with 5-ASA.

3. Introduction

The human intestine is colonized by a huge number of microorganisms, chiefly in its distal portion, commonly called microbiota or commensal flora [1]. The relationship established between the host and the commensal flora is of a symbiotic nature, since, for instance, the microbiota increases the digestion capacity of the host and helps him in preventing pathogen colonization, benefiting, in turn, from both the nutrients and the warm temperature of the human body [1-3]. However, given the close proximity between the human intestine (with a surface area of approximately 200 m^2) and the resident microorganisms and its constant exposure to pathogenic agents from the diet, the intestinal epithelium has devised strategies to avoid microbial breach and to assure intestinal homeostasis [1]. This is accomplished thanks to the intestinal epithelium's ability to function as an efficient barrier to be underpinned by its capacity of triggering adequate immune responses against potential pathogens-derived signals [4-6]. Intestinal epithelial cells as well as immune cells express pattern-recognition receptors (PRRs), which after detecting microbial-associated molecular patterns (MAMPs), namely lipopolysaccharide and flagellin, up-regulate a panoply of pro-inflammatory signalling cascades, leading to the transcription of several inflammatory genes, to the production and release of many inflammatory mediators and to the generation of reactive oxygen and nitrogen species (ROS/RNS) [2, 7, 8]. Hence, cell inflammation and oxidative reactions as self-limited processes are involved in the orchestration of innate and adaptive immune responses in order to eradicate potential pathogens [9]. However, a persistent induction of intestinal inflammation with excessive production of reactive species can become pathological, as in the case of the well-known chronic inflammatory disorder of the gastrointestinal tract, the Inflammatory Bowel Disease (IBD) [9-11]. Although the etiology of IBD remains uncertain, it is known that an interplay between genetic predisposition, due, for instance, to mutations in nucleotide-binding oligomerization domain containing 2 (*NOD2*) gene, and environmental factors, such as stress, diet and antibiotic intake, provokes a deregulation of the immune reaction to dietary antigens or commensal microorganisms, culminating in the perpetuation of uncontrolled and exacerbated inflammatory and oxidative responses [3, 11, 12].

Dietary polyphenols can be considered excellent candidates as a complementary nutritional approach to treat chronic inflammatory diseases, as IBD, due to the growing wealth of information demonstrating their potent antioxidant capacity allied to their beneficial anti-inflammatory effect in many *in vitro* and *in vivo* models [13-16]. Furthermore, although

the low oral bioavailability of the majority of polyphenols can constitute a great handicap for its use in the context of several diseases [17], in the particular case of IBD, the poor oral bioavailability is not a true problem, since several studies have suggested that the intestine is the organ where dietary polyphenols possibly achieve the highest concentrations in the human body [10, 18, 19]. On the other hand, it has been reported that these compounds can modulate intestinal inflammatory response, which is an important component of IBD pathogenesis. In this context, several cell signalling pathways have been proposed as potential targets of polyphenols protective activity against intestinal inflammation and oxidative insult [18]. Nuclear factor erythroid 2 (Nrf2) signalling pathway is considered the most important cellular defence against oxidative stress [20], being also important in the suppression of the inflammatory process by counteracting some pro-inflammatory cascades, as nuclear factor kappa B (NF-kB) pathway, among other mechanisms [21]. Also, the peroxisome proliferator-activated receptor gamma (PPAR-γ), a nuclear receptor, was recently considered important for inhibiting intestinal inflammatory response and defending cells from oxidative damage [22, 23].

Among polyphenols, cyanidin-3-glucoside (C3G) and resveratrol (Resv) (**Figures 1A and B**, respectively) are widespread in the human diet, particularly in fruits, vegetables and red-wine, with recognized beneficial health effects [24]. The interest in the study of these polyphenols arose, at least in part, from their relatively high contents in red-wine, which is very much appreciated by Western Societies [10, 17]. Recently, we have shown that both polyphenolic compounds have a more efficient anti-inflammatory activity than 5-aminosalicylic acid (5-ASA), a well-known anti-inflammatory drug, commonly used in the treatment of IBD (**Figure 1C**), by decreasing the production of inflammatory markers, via down-regulation of cytokine-induced Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway, in a colonic epithelial cell line [13, 14].

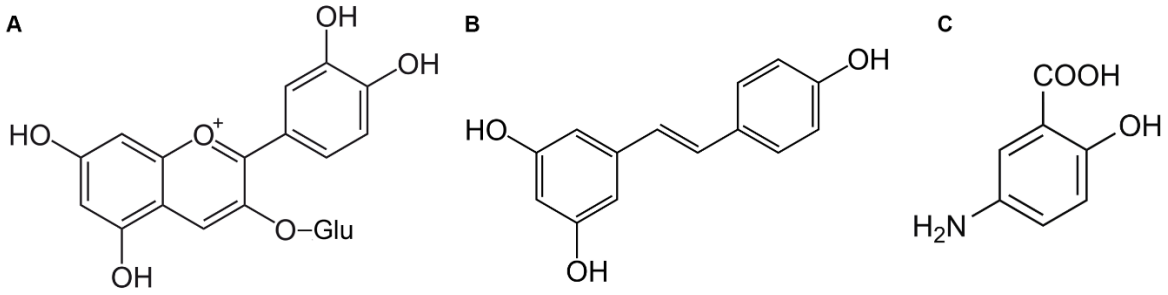


Figure 1 - Chemical structures of cyanidin-3-glucoside (A), resveratrol (B) and 5-aminosalicylic acid (C).

To give continuity to the work previously developed and considering that inflammation and oxidative stress are crucial close related phenomena in the pathogenesis of IBD [11], the present study was conducted to elucidate the ability of these polyphenols to induce antioxidant and detoxifying cellular defences, via Nrf2 up-regulation, in comparison with 5-ASA, by using the same *in vitro* model, the HT-29 cell line and the same experimental conditions. On the other hand, since 5-ASA has been suggested to be an agonist of PPAR-γ, relying its anti-inflammatory activity on PPAR-γ pathway [25], another goal of this work was to scrutinize the involvement of this pathway in the protection afforded by either C3G or Resv against cytokine-induced inflammatory and oxidative events in HT-29 cells, as compared to 5-ASA.

4. Materials and Methods

4.1 Reagents

Cyanidin-3-glucoside (C3G) and resveratrol (Resv) purified from natural sources were obtained from Extrasynthése (Genay, France). Their purity were above 95%, as measured by HPLC, and they were used as a solution in DMSO (5 mM) and stored at -20 ºC.

Laboratory chemicals, namely, dimethylsulfoxide (DMSO), 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), phenylmethylsulfonyl fluoride (PMSF), streptomycin/penicillin, protease inhibitor cocktail, phosphatase inhibitors and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Co.

For cell culture, Dulbecco's modified Eagle's medium (DMEM), 0.25% trypsin, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) pH 7.4, were obtained from Gibco-Invitrogen.

Mouse monoclonal antibody to PPAR-γ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and rabbit polyclonal antibody to lamin B1 was obtained from Abcam (Cambridge, UK).

IL-1α, TNF-α and IFN-γ were purchased from Invitrogen (NY, USA).

4.2 Cell Culture

Human colon cancer cell line (HT-29) was obtained from European Collection of Cell Cultures (Porton Down, Salisbury, UK). Cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37ºC in a humidified atmosphere of 5% CO_2. Cells were sub-cultured at confluence and used between the fourth and the twentieth passage. Before each experiment, cells at 80% confluence were starved in serum-free medium for 24 hours. Growth-arrested cultures, in medium without FBS, were treated according to the various experimental purposes.

HT-29 cells were stimulated with a cocktail of cytokines consisting of 10 ng/ml IL-1α, 20 ng/ml TNF-α and 60 ng/ml IFN-γ. Each cytokine was previously diluted in PBS with 1% bovine serum albumin (BSA) and then added to cells when convenient. Cells were pre-incubated with 25 µM C3G or 25 µM Resv*per se* or in combination with 500 µM 5-ASA, for 1 hour and then exposed to the pro-inflammatory cytokines for different time points, depending on the assay. The concentrations of polyphenols and 5-ASA were selected taking into consideration the range

of concentrations referred in the literature as being reached at the intestinal level [10, 18, 26].

4.3 Evaluation of Nrf2 DNA Binding Activity

DNA binding activity of Nrf2 was measured in nuclear extracts using the TransAM^{TM} Nrf2 protein assay (Active Motif, CA, USA), an ELISA-based method with high sensitivity and reproducibility.

For preparation of nuclear extracts, washed cells were lysed in an ice-cold buffer containing 10 mM Tris–HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 and 1% protease inhibitor cocktail, pH 7.5, for 5 minutes on ice. Afterwards, lysates were centrifuged at 5000 rpm for 5 minutes at 4ºC and the pellets were collected and resuspended in 50 µl of complete lysis buffer (a solution provided by Active Motif, CA) and left on ice for 30 minutes. Then, lysates were centrifuged at 14000 rpm for 20 minutes at 4ºC and the supernatants (nuclear extracts) were saved at -80ºC.

DNA binding activity of Nrf2 was evaluated in 20 µg of nuclear protein, according to the manufacturer's protocol and the results expressed in relative terms.

4.4 Total RNA Extraction and quantitative Real-Time RT-PCR (qRT-PCR)

Total RNA was extracted from HT-29 cells seeded in six-well-plates (8 x 10^5 cells/well) by using the RNA extraction kit Aurum^{TM} Total RNA Mini (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Extracted RNA was quantified using a NanoDrop ND-1000 spectrophotometer at 260 nm and its purity and integrity were assessed by the ratio of absorbances at 240/260 and 280/260 nanometers. A constant amount of RNA (1 µg/sample) was reverse transcribed into cDNA, using the NZY First-Stand cDNA Synthesis Kit (NZYtech, Portugal), according to the manufacturer's protocol. PCR reactions were performed with 25 µg/ml of transcribed cDNA. The primers for the HO-1, the catalytic and modifier subunits of glutamate cysteine ligase (GCLC and GCLM, respectively) and the housekeeping gene HPRT-1 (hypoxanthine phosphoribosyltransferase-1) were designed using the Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA) and the primers sequences were: HO-1, sense 5´- TCACTGTGTCCCTCTCTC -3', antisense 5'- ATTGCCTGGATGTGCTTT -3'; GCLC, sense 5'- ATTCTGAACTCTTACCTTGA -3'; antisense 5'- ATCTGGCAACTGTCATTA -3'; GCLM, sense

5’- AACTCTTCATCATCAACTA -3’; antisense 5’- AACTCCATCTTCAATAGG -3’ and HPRT-1, sense 5’- TGACACTGGCAAAACAATG -3’, antisense 5’- GGCTTATATCCAACACTTCG -3’. Real time-PCR was performed in 20 µl of total volume, containing 2 µl of each primer (250 nM), 2 µl of cDNA of each sample, 10 µl of the IQ ™ SYBR Green Supermix (Bio-Rad) and RNase-free distilled water to make up the volume to 20 µl, in a CFX96 ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Thermal cycling conditions were the following: 3 minutes at 95 ºC to activate the iTaq™ DNA polymerase, then 45 cycles, each consisting of a denaturation step (95ºC, 10 seconds), an annealing step (55ºC, 30 seconds) and an elongation step (72ºC, 30 seconds). Fluorescence measurements were taken every cycle at the end of the annealing step and the specificity of the amplification products was evaluated through the analysis of the melting curve. The efficiency of the amplification reaction for each gene was calculated by running a standard curve of serially diluted cDNA sample. Gene expression was analysed using the Bio-Rad CFX Manager 3.0 software (Bio-Rad, Hercules, CA, USA), which enables the analysis of the results with the Pfaffl method. The results for each gene of interest were normalized against HPRT-1, the housekeeping gene found to be stable under experimental conditions and expressed as a percentage of control cells, *i.e.* non-stimulated cells.

4.5 Measurement of Reduced (GSH) and Oxidized Forms of Glutathione (GSSG)

The intracellular contents of GSH and GSSG were determined by a fluorimetric assay, as described by Hissin and Hilf [27]. Briefly, GSH and GSSG are measured upon their reactions with a fluorescent reagent, o-phthaldialdehyde (OPT), at pH 8 or pH 12, respectively. Before GSSG determination, GSH is complexed to N-ethylmaleimide (NEM) to prevent its oxidation and consequently its interference with GSSG quantification.

After 24 hours of incubation, cells were washed twice with cold PBS, detached and resuspended in 500 µl of 100 mM Na_2HPO_4, pH 8. After addition of an equal volume of 0.6 M $HClO_4$, the mixture was maintained on ice for 5 minutes. After vigorous vortex, the cellular extracts were centrifuged at 14000 rpm for 5 minutes, at 4ºC. Supernatants were collected and the respective pellets were resuspended in 1 M NaOH for protein quantification. For GSH quantification, 100 µl of each sample was added to 1800 µl of 100 mM Na_2HPO_4, pH 8 buffer, and to 100 µl of OPT and maintained in the dark, at room temperature, for 15 minutes, before

fluorescence detection. A standard curve was also prepared with known concentrations of GSH. For GSSG quantification, NEM was added to an aliquot of each sample for 30 minutes to complex GSH, and the following protocol was similar to that mentioned above to GSH measurement, but using 100 mM NaOH instead of Na_2HPO_4, to carry out the reaction at pH 12. Fluorescence intensity was read in a Synergy HT plate reader (Bio-Tek Instruments) (excitation and emission wavelengths at 350 and 420 nm, respectively). Cellular GSH and GSSG contents were calculated using concurrently run standard curves and expressed as nmol GSH or GSSG per milligram of protein. Cellular protein was determined by using the Bio-Rad protein assay reagent (Bradford assay), according to the manufacturer's specifications (Bio-Rad, USA).

4.6 Evaluation of Intracellular Reactive Species

The production of intracellular reactive species were assessed by using the non-fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), which permeates cell membranes and may be deacetylated by cellular esterases and rapidly oxidized by reactive species, yielding the fluorescent 2′,7′-dichlorofluorescein (DCF) [28]. Briefly, cells in 12-well plates (4 x 10^5 cells/well) were previously incubated in the presence or in the absence of C3G and 5-ASA and further subjected to the combination of cytokines for 24 hours. After that period of time, the cells were incubated with 5 μM $DCFH_2$-DA in DMSO, at 37°C, in the dark for 15 minutes. Cells were then washed with PBS and maintained in 0.5 ml of PBS during the fluorescence intensity measurements in a Synergy HT plate reader (Bio-Tek Instruments) (excitation and emission wavelengths at 485 and 530 nm, respectively). Cells were also observed in an inverted fluorescence microscope (Zeiss Axiovert 40), using a FITC filter.

4.7 Western-blot Analysis

As previously described [13], nuclear cellular protein extracts from HT-29 cells were prepared and analysed by Western-blotting. Cells were washed with cold PBS and then lysed in an ice-cold buffer containing 10 mM Tris–HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 and 1% protease inhibitor cocktail, pH 7.5, for 5 minutes on ice. Afterwards, lysates were centrifuged at 5000 rpm for 5 minutes at 4ºC and the pellets were collected and resuspended in an ice-cold buffer with 20 mM Hepes, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 300 mM NaCl, 20% (w/v) glycerol, and 1% protease inhibitor cocktail, pH 7.5 and left on ice for 30

minutes. Then, the mixture was centrifuged at 14000 rpm for 20 minutes at 4ºC and the supernatants, the nuclear extracts, were saved at -80ºC.

Protein concentration was determined by using the Bio-Rad protein assay reagent (Bradford assay), according to the manufacturer's specifications (Bio-Rad, USA). Fifty micrograms of reduced and denatured nuclear proteins were separated by SDS/PAGE electrophoresis on a 10% (v/v) acrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, UK) by electroblotting. To avoid non-specific binding, membranes were blocked with skimmed milk in TBS pH 7.6 supplemented with 0.1% (v/v) Tween 20 (TBS-T: 20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) and then probed with the antibody against PPAR-γ, overnight at 4ºC, with a constant low shaking. After finishing, membranes were washed three times and further incubated with the alkaline phosphatase-conjugated secondary antibody (2 hours at room temperature and constant shaking). Immunoreactive bands were detected by fluorescence in a Typhoon 9000 scanner (Amersham Biosciences) and analysed with the ImageQuant TM software from Amersham Biosciences. After analysis of the target protein, each blot was stripped off and reprobed with the primary antibodies against lamin B1, used as control for nuclear protein loading.

4.8 Statistical Analysis

All data were expressed as means ± SEM of at least 3 independent assays, each one in duplicate. Differences between groups were analysed by one-way analysis of variance (ANOVA) and Tukey's *post hoc* test was used as appropriate. Values of $p<0.05$ were accepted as statistically significant.

5. Results

5.1 Either C3G or Resv induced the activation of Nrf2, in cytokine-stimulated HT-29 cells

Nuclear factor erythroid 2 (Nrf2) is a redox-sensitive transcription factor with a crucial role in counteracting the overproduction of reactive oxygen species as well as in inhibiting inflammation. Therefore, to progress our study of the mechanisms underlying the anti-inflammatory activity of C3G and Resv in comparison with 5-ASA, we intended to elucidate about their effects on this key transcription factor.

So, in the present study, we started to ascertain the effect of a group of cytokines used as inflammatory stimulus on the modulation of Nrf2 activation, over time, as described in materials and methods. As shown in **Figure 2A**, Nrf2 DNA binding activity increased approximately twofold, compared to the levels in control cells (untreated cells) and remained enhanced up to 8 hours of cytokine challenge. However, after 16 hours of cytokine exposure, DNA binding activity of Nrf2 was similar to that of non-stimulated cells (control cells).

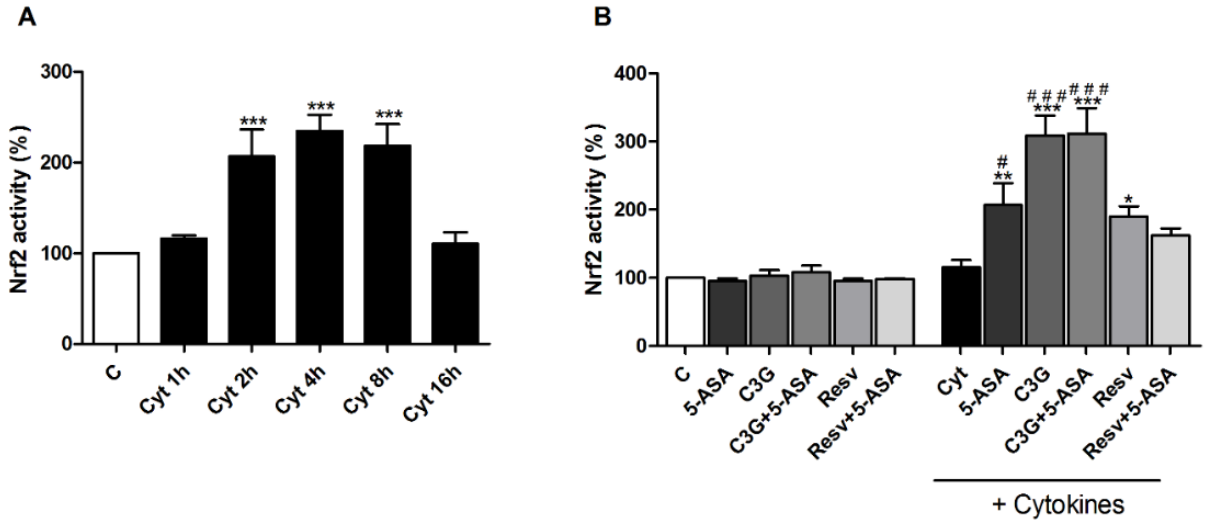


Figure 2 – C3G and Resv increase Nrf2 activation in cytokine-stimulated HT-29 cells, in a more efficient way than 5-ASA. In 2A, cells were incubated with a combination of pro-inflammatory cytokines (Cyt) and the Nrf2 DNA binding activity was evaluated over time. In 2B, cells were pre-incubated with 25 µM C3G, 25 µM Resv, 500 µM 5-ASA or with combinations of each polyphenol with 5-ASA, for 1 hour, and then exposed to a cocktail of cytokines for 16 hours. The Nrf2 activation in cells was measured as described in "Materials and Methods" and expressed as percentage of control cells (C) (non-stimulated cells). Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs Control cells, $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs Cytokines-stimulated cells.

Being aware that after 16 hours of cytokine-stimulation, this mixture of cytokines was no longer able to produce an increase in Nrf2 DNA binding activity, we investigated, at that time, the effect of C3G and Resv on Nrf2 DNA binding activity, in comparison with 5-ASA, in cells challenged or not challenged with the cytokine mixture. As depicted in **Figure 2B**, C3G and Resv *per se*, and the combination of C3G plus 5-ASA were successful in promoting the activation of Nrf2, in cytokine-exposed cells, similarly to 5-ASA. However, the stimulatory effect of C3G alone was clearly and statistically higher than that assigned to 5-ASA, particularly if the difference in the concentrations of the compounds are taken into account (25 µM C3G versus 500 µM 5-ASA). As compared to C3G, Resv showed a much lower activation effect of Nrf2, but also higher than that of 5-ASA, considering the difference in the concentrations (25 µM Resv versus 500 µM 5-ASA). Of note is that the combination of 5-ASA with C3G or Resv did not increase those observed effects in cytokine-stimulated HT-29 cells. In cells not exposed to cytokines (control cells), DNA binding activity of Nrf2 was not induced neither by polyphenols, 5-ASA nor their combinations.

5.2 C3G and Resv, unlike 5-ASA, up-regulated HO-1 mRNA expression, in cytokine-stimulated HT-29 cells

In pursuit of knowing more about the beneficial effects of C3G and Resv in cytokine-stimulated HT-29 cells, we explored the ability of these polyphenols to modulate the expression of some Nrf2 target genes. Therefore, we evaluated the effect of those compounds in one ARE-regulated cytoprotective enzyme, the hemoxygenase-1 (HO-1), at a transcriptional level, in comparison with 5-ASA, by performing qRT-PCR (**Figure 3**).

In this figure, it is observed that after a stimulation period (20 hours) of HT-29 cells with cytokines, the HO-1 mRNA levels were statistically similar to those in non-stimulated cells (control). The pre-treatment of control cells with the compounds under study, either alone or in combination with 5-ASA, in similar conditions, did not modify the HO-1 mRNA levels. Nevertheless, it is clear that in cytokine-stimulated cells, the pretreatment with either C3G or Resv induced *per se* a significant increase in the HO-1 mRNA expression. No additional remarkable effect was obtained with the pretreatment with C3G plus 5-ASA. In contrast with C3G alone, the pre-treatment with 5-ASA alone or in combination with Resv did not induce any increase in HO-1 mRNA levels, in cytokine-exposed cells. Therefore, it is worth of notice the very high efficiency of C3G and Resv in up-regulating HO-1 mRNA expression, an effect

not assigned to 5-ASA, in a concentration 20 times higher.

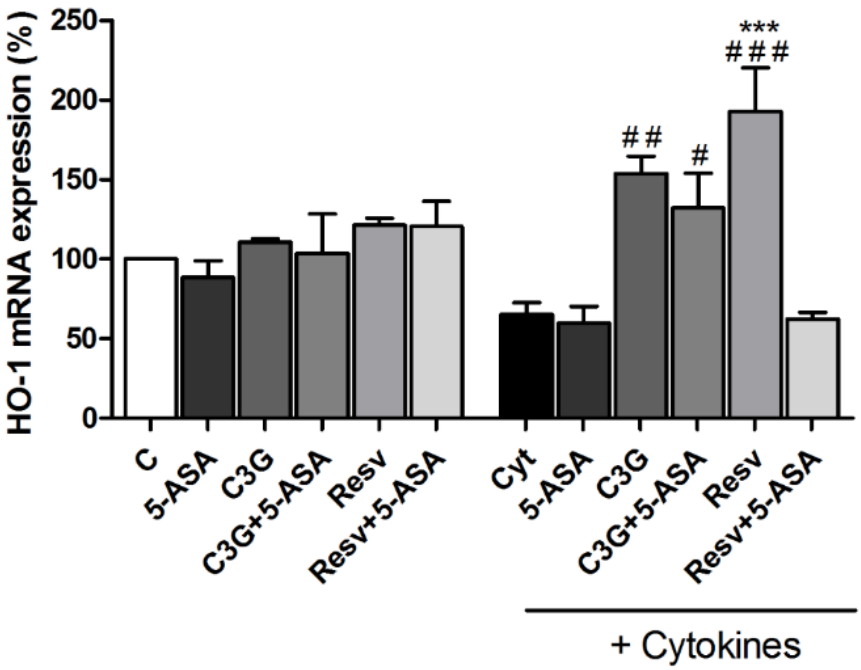


Figure 3 – C3G and Resv, unlike 5-ASA, enhance mRNA expression of HO-1 in cytokine-stimulated HT-29 cells. Cells were pre-incubated with 25 µM C3G, 25 µM Resv, 500 µM 5-ASA or with combinations of each polyphenol with 5-ASA and then exposed to a cocktail of cytokines for 20 hours. The mRNA production of HO-1 was evaluated by qRT-PCR, as described in "Materials and Methods", and expressed as percentage of control cells. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{***}P<0.001$ vs Control cells, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs Cytokines-stimulated cells.

5.3 C3G and Resv increased mRNA expression of catalytic and modifier subunits of GCL, in cytokine-stimulated HT-29 cells, in a much lower concentration than 5-ASA

Glutamate cysteine ligase (GCL) is another ARE-regulated cytoprotective enzyme crucial for cellular defence against oxidative stress, by promoting the first-step of the synthesis of intracellular glutathione. The expressions of both GCL catalytic and modifier subunits (GCLC and GCLM, respectively) were evaluated, at a transcriptional level, by qRT-PCR, in HT-29 cells challenged or not with a mixture of cytokines for 20 hours and pre-treated or not with C3G, Resv and/or 5-ASA. As patent in **Figure 4**, the expression of GCLC remained statistically unchanged after cytokine-exposure (**Figure 4A**), conversely to GCLM, whose expression slightly increased in cytokine-stimulated cells (**Figure 4B**).

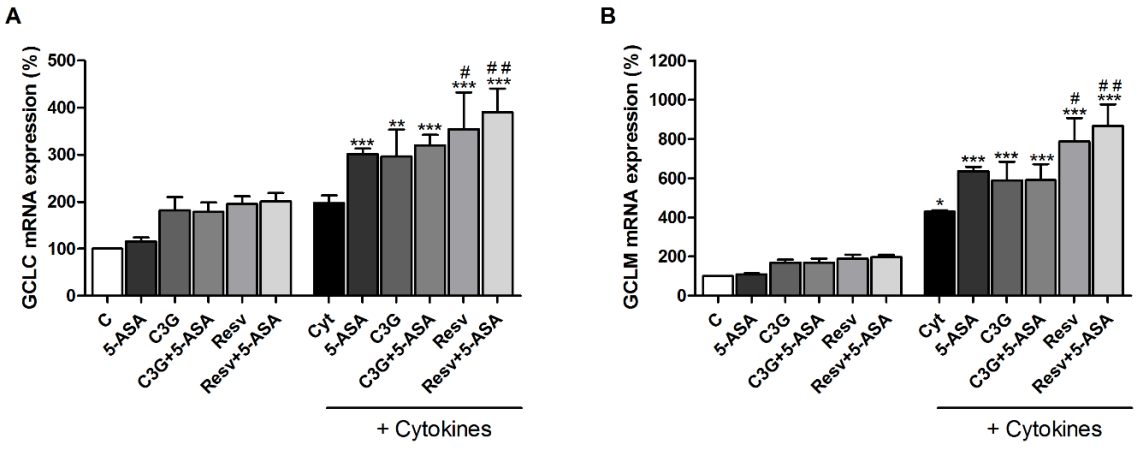


Figure 4 – C3G and Resv promote mRNA expression of GCLC and GCLM in cytokine-stimulated HT-29 cells, in a much lower concentration than 5-ASA. Cells were pre-incubated with 25 µM C3G, 25 µM Resv, 500 µM 5-ASA or with combinations of each polyphenol with 5-ASA and then exposed to a cocktail of cytokines for 20 hours. The mRNA production of both catalytic and modifier subunits of GCL (GCLC (4A) and GCLM (4B)) was evaluated by qRT-PCR, as described in "Materials and Methods", and expressed as percentage of control cells. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs Control cells, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs Cytokines-stimulated cells.

The pre-treatment of these cells with either C3G or Resv, alone or in combination with 5-ASA, before cytokine exposure, triggered a significant increase in the expression of both GCLC and GCLM, as compared to control cells, like with 5-ASA, but at a concentration 20 times lower (**Figures 4A** and **4B**). The association of these polyphenols with 5-ASA did not significantly improve the effects of C3G and Resv alone. In cells not exposed to the inflammatory stimulus (control cells), the treatment with those compounds, in similar conditions, did not change significantly the expressions of GCL subunits (**Figures 4A** and **4B**).

5.4 Unlike 5-ASA, C3G and Resv enhanced the GSH/GSSG ratio, in cytokine-exposed HT-29 cells

Considering that C3G and Resv significantly increased the expression of GCL in cytokine-stimulated cells and that this enzyme is a major determinant of cellular glutathione levels, we evaluated the effects of those polyphenols on the reduced glutathione to oxidized glutathione ratio (GSH/GSSG ratio), in comparison with 5-ASA. As illustrated in **Figure 5**, the GSH/GSSG ratio did not undergo significant changes 24 hours after cytokine stimulation, remaining similar to that of unstimulated cells (control). However, in cytokine-exposed cells, the pre-incubation with 25 µM C3G or Resv alone resulted in a significant enhancement of the GSH/GSSG ratio. Similar results were obtained with the pre-incubation of these cells with Resv plus 5-ASA, but, on the contrary, neither the incubation with 5-ASA alone nor its combination with C3G was able to increase the GSH/GSSG ratio. In non-stimulated cells, the incubation with those compounds, in similar conditions, did not change the reduced/oxidized glutathione ratio.

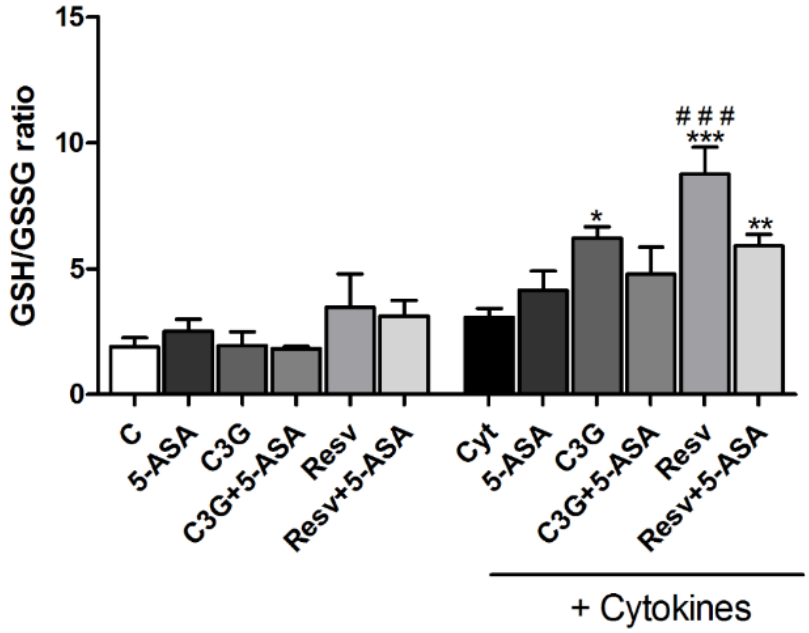


Figure 5 – C3G and in particular Resv enhance the GSH/GSSG ratio in cytokine-stimulated HT-29 cells, much more efficiently than 5-ASA. Cells were pre-incubated with 25 µM C3G, 25 µM Resv, 500 µM 5-ASA or with combinations of each polyphenol plus 5-ASA and then exposed to a cocktail of cytokines for 24 hours. Cellular content of GSH and GSSG was measured by fluorescence intensity, as described in "Materials and Methods" and expressed in nmol of glutathione/mg protein. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs Control cells, $^{\#\#\#}P<0.001$ vs Cytokines-stimulated cells.

5.5 C3G, unlike 5-ASA, provoked a strong inhibition of intracellular reactive species production, in cytokine-stimulated HT-29 cells

It is well-known that in response to chronic inflammation, the overproduction of intracellular reactive species occurs, and subsequently, several redox-sensitive transcription factors remain up-regulated, leading to the generation of more and more intracellular reactive species. As recently reported by us, this mixture of cytokines significantly increased reactive species production, as evaluated by dichlorodihydrofluorescein fluorescence, in a time-dependent manner [14]. Besides, in cytokine-stimulated cells, we have shown that the pre-incubation with Resv, either alone or in combination with 5-ASA, inhibited the formation of reactive species more efficiently than 5-ASA [14]. In this sequence, the potential protective effect of C3G was assessed, by using the same methodology. In agreement with the data previously reported by us [14], the exposure of HT-29 cells to the selected cocktail of cytokines for 24 hours, conducted to a significant increase in the intracellular levels of reactive species. As shown in the bar graph and in the top pictures of **Figure 6**, C3G alone exhibited a higher efficiency than 5-ASA in inhibiting intracellular reactive species production, particularly taking into account the difference in the concentrations used (25 µM C3G and 500 µM 5-ASA). The association of C3G plus 5-ASA did not significantly protect against reactive species formation, in cytokine-exposed cells.

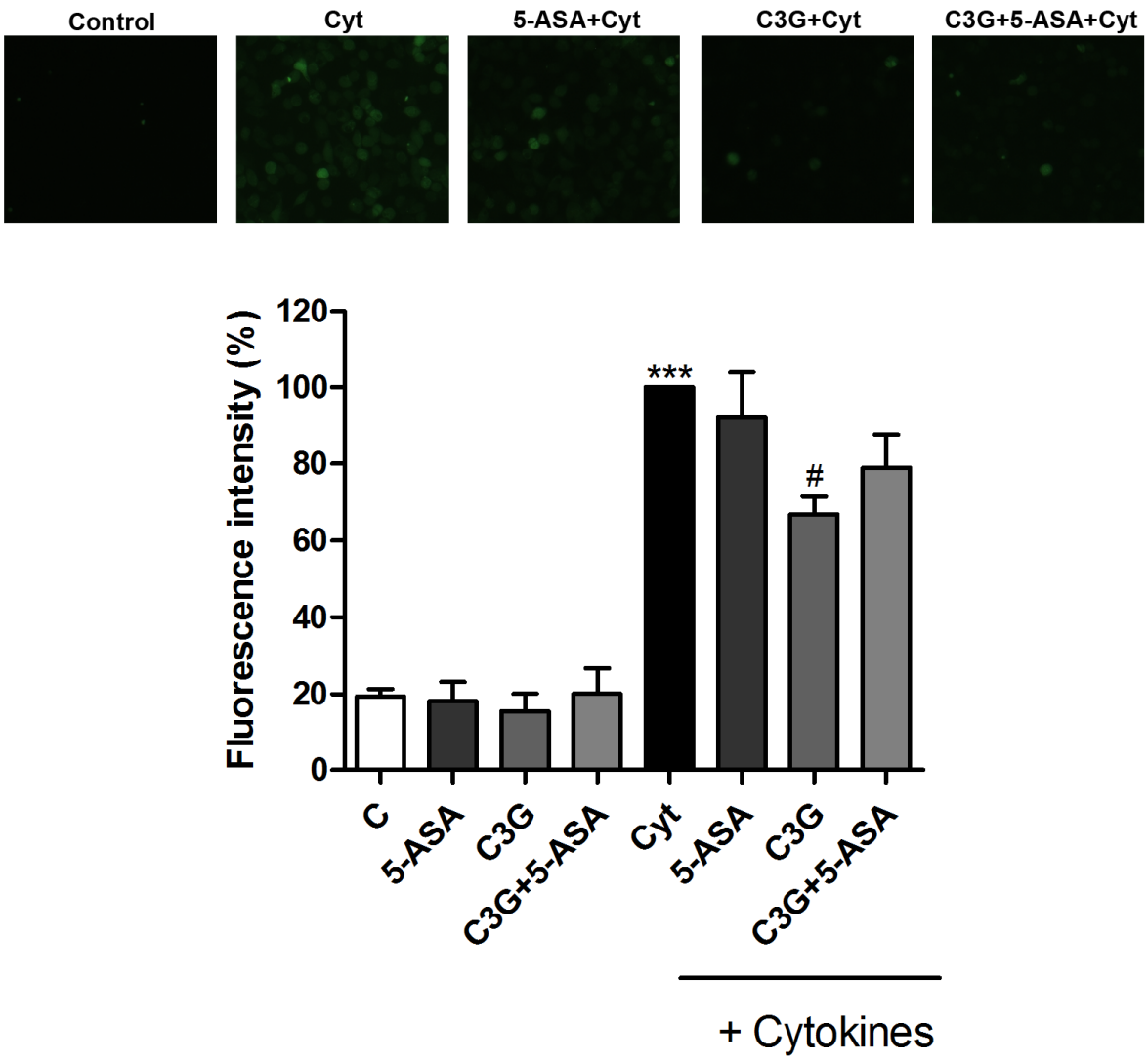


Figure 6 – C3G inhibits reactive species generation in cytokine-stimulated HT-29 cells, more efficiently than 5-ASA. Cells were pre-incubated with either 25 µM C3G, 500 µM 5-ASA or both 25 µM C3G plus 500 µM 5-ASA for 1 hour and then exposed to a cocktail of cytokines for 24 hours. Intracellular reactive species generation was measured by oxidation of the probe dichlorodihydrofluorescein and expressed in terms of fluorescence intensity relative to cytokine-stimulated HT-29 cells. Representative images obtained by fluorescence microscopy (400x) of cells at 24 hours after cytokine treatment, in the absence or presence of 25 µM C3G and/or 500 µM 5-ASA, are presented on the top. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{***}P<0.001$ vs Control cells, $^{\#}P<0.05$ vs Cytokines-stimulated cells.

5.6 Resv, unlike C3G, increased the expression of PPAR-γ in the nucleus of cytokine-challenged HT-29 cells, similarly to 5-ASA, but in a much lower concentration

Allying the knowledge about the involvement of peroxisome proliferator-activated receptor gamma (PPAR-γ) in the inhibition of both intestinal inflammation and oxidative events with some work suggesting that the anti-inflammatory action of 5-ASA is dependent on PPAR-γ [25], we attempted to evaluate the effects of C3G and Resv on the expression of this transcription factor in the nucleus of HT-29 cells, in comparison with 5-ASA. As demonstrated in **Figure 7**, none of the compounds in study was able to increase significantly the expression of PPAR-γ in the nucleus of cells not exposed to cytokines. However, in cells stimulated with the cytokines cocktail, Resv *per se* was able to increase the nuclear expression of this transcription factor in a similar extent to 5-ASA, but in a concentration 20 times lower. On the contrary, C3G did not enhance the nuclear expression of PPAR-γ, suggesting that this transcription factor is not involved in the protection afforded by this anthocyanin against the cytokine-induced inflammatory process in HT-29 cells.

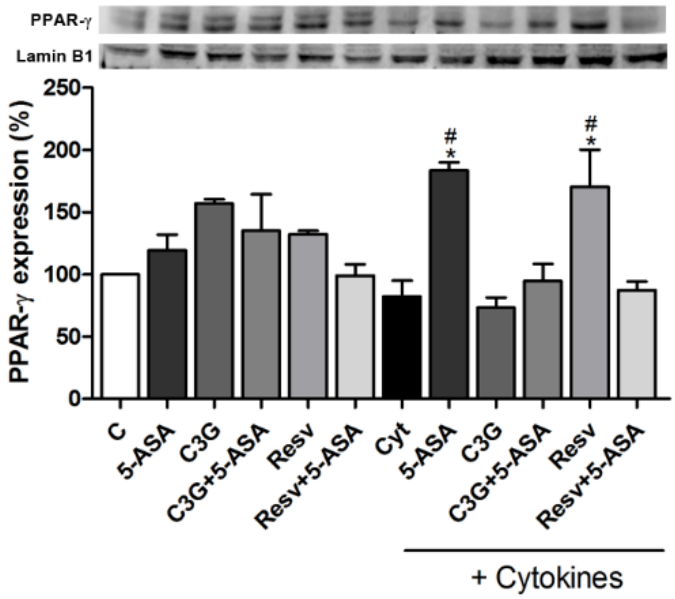


Figure 7 - Unlike C3G, Resv increases the nuclear accumulation of PPAR-γ in cytokine-stimulated HT-29 cells, similarly to 5-ASA, but in a much lower concentration. Cells were pre-incubated with either 25 µM C3G, 25 µM Resv, 500 µM 5-ASA or with combinations of each polyphenol with 5-ASA (25 µM C3G/Resv plus 500 µM 5-ASA) and then exposed to a cocktail of cytokines for 16 hours. PPAR-γ expression was evaluated in nuclear extracts by Western blotting, as described in "Materials and Methods", and expressed as percentage of control cells. Values are mean ± SEM of at least three independent experiments, each one in duplicate. $^{*}P<0.05$ vs Control cells and $^{\#}P<0.05$ vs Cytokines-stimulated cells.

6. Discussion

In spite of the etiology of Inflammatory Bowel Disease (IBD) remains unclear, it is widely accepted that inflammation and oxidative stress play a pivotal role in the pathogenesis of this disease [11].

In the last decades, a flourishing research field has been focused on the study of the antioxidant and anti-inflammatory activities of several dietary polyphenols, suggesting that these phytochemical compounds can be envisaged as promising agents in the prevention and treatment of several chronic inflammatory disorders, as IBD [16, 18]. However, the precise cellular signalling mechanisms and targets involved in their protective effects are still under debate.

Nrf2 pathway has emerged as a key regulator of cellular antioxidant and detoxifying defences against oxidative insult, requiring a proper regulation to assure the intestinal homeostasis [20]. Under basal conditions, Nrf2 is sequestered in the cytoplasm, bound to the inhibitory protein Kelch-like ECH-associated protein 1 (Keap1), which has the ability to target Nrf2 for proteasomal degradation [29]. After activation, it is known that Nrf2 translocates to the nucleus, heterodimerizes with other proteins, namely small Maf proteins, and binds to antioxidant response elements (ARE) in enhancer sites of responsive genes, promoting the transcription of several important antioxidant and cytoprotective enzymes, namely hemoxygenase-1 (HO-1) and glutamate cysteine ligase (GCL) [21]. Beyond the anti-oxidative protection afforded by Nrf2 activation, this transcription factor has demonstrated to be also crucial in the suppression of the inflammatory process, namely by down-regulating some important pro-inflammatory cascades, such as NF-kB [21]. In line with this, accelerating research has suggested that Nrf2 pathway is essential to counteract dextran sulfate sodium (DSS)-induced gut inflammation in mice [30] and, for this reason, the potential of dietary polyphenols to up-regulate Nrf2 pathway has gained attention in the context of IBD.

Following our previous report showing that a mixture of cytokines (IL-1α, TNF-α and IFN-γ) in HT-29 cells induced the expression and activation of two relevant pro-inflammatory cascades, NF-kB and STAT1 [13], in the present study, a start aim was to investigate the effect of that cytokine mixture on the regulation of Nrf2 pathway. We demonstrated, for the first time, that the exposure of intestinal cells to a group of pro-inflammatory cytokines led, in the beginning, to a growing induction of Nrf2 transcriptional activation (up to 8 hours). It is possible to speculate that this increase of Nrf2 transcriptional activation may constitute a

homeostatic mechanism in order to counteract the inflammatory response and the excessive production of reactive species. This finding is consistent with the study of Rushworth *et al* [31] showing the ability of TNF-α to induce a prolonged Nrf2 activation in monocytes. However, in our experimental conditions, Nrf2 transcriptional activity returned to basal levels after 16 hours of cytokines exposure, suggesting that intestinal cells were no longer able to hamper the inflammation and oxidative stress, at least through Nrf2 pathway, after that time. This assumption is strengthen by our previous work, showing that intestinal cells expressed high levels of several pro-inflammatory markers (namely, COX-2 and PGE_2) [13] as well as increasingly amounts of reactive species [14], 16 hours after cytokines exposition.

Taking into account that after this stimulation period, the Nrf2 activity recovered the basal levels, we investigated, at this time point, the effects of C3G and Resv on the regulation of the activation of this transcription factor, as compared to 5-ASA. We observed that C3G and Resv, in a concentration 20 times lower than 5-ASA, significantly increased Nrf2 activity in cytokine-stimulated cells, prolonging its activation. This is in accordance with a recent study by Speciale *et al* [32], indicating that C3G was able to increase the translocation of Nrf2 to the nucleus of TNF-α-stimulated human umbilical vein cells (HUVECs), which is a very good indicator of Nrf2 activation. Moreover, Hao *et al* [33] have also reported that Resv showed similar effects in lipopolysaccharide-exposed cultured primary human cardiomyocytes. It is worth to note that, in our study, the combinations of C3G or Resv with 5-ASA did not improve the effects obtained with each polyphenol *per se*, demonstrating no additive or synergistic effects.

Since C3G and Resv were able to promote the transcriptional activity of Nrf2, we assessed the effects of these dietary polyphenols on the expression of two ARE-regulated enzymes, HO-1 and GCL, as compared to 5-ASA. HO-1 is the inducible isoform of the hemoxygenase enzyme that catalyses the oxidation of heme to carbon monoxide (CO), iron and biliverdin [34]. In our experimental conditions, and in contrast to 5-ASA, C3G and Resv significantly increased the expression of HO-1 in cytokine-challenged cells, at a transcriptional level. This finding can be seen as a point in favour of the potential use of the polyphenols in the detriment of 5-ASA, since the up-regulation of this enzyme has demonstrated to contribute to ameliorate experimentally-induced colitis [35, 36]. Besides, as occurred with Nrf2 activation, the combination of C3G or Resv with 5-ASA did not represent an advantage over the use of individual compounds to promote the HO-1 expression. Although the mechanisms behind the protective effect of HO-1 on colitis are not fully understood, some recent studies indicated that

this enzyme interferes with the expression of a crucial pro-inflammatory enzyme, the inducible nitric oxide synthase (iNOS), which could be a putative molecular mechanism underlying, at least in part, the beneficial effects of some HO-1 inducers, as reported in both *in vitro* and *in vivo* studies [35, 37]. Thus, strikingly, the increments of HO-1 expression promoted by the pre-incubation of either C3G or Resv in cytokine-stimulated cells may be related to their abilities to inhibit the expression of iNOS and the consequent production of nitric oxide (NO) in those cells, as previously reported by us [13, 14]. Concerning to GCL, this enzyme is responsible for the first limiting-step of the biosynthesis of the glutathione (GSH) and it comprises a catalytic subunit (GCLC), with all the enzymatic activity, and a modifier subunit (GCLM), which is enzymatically inactive but with capacity of increasing the catalytic efficiency of GCLC [38]. The GSH is a tripeptide recognized as the most important intracellular antioxidant, which can be oxidized to GSSG, by ROS/RNS, being the ratio GSH/GSSG an optimal indicator of the intracellular redox state [39]. Regarding to our main findings, it is important to highlight two aspects: firstly, the cytokine stimulation of HT-29 cells did not alter the expression of GCLC as compared to non-stimulated cells, whereas it enhanced GCLM expression in the same cells. Although there is some controversy around this issue, Franklin *et al* [40] have reported that the enhancement of GCLM expression, even not accompanied by an increment of GCLC, is an effective mechanism for increasing GCL activity. However, in our study, the levels of GSH did not suffer any change in cytokine-stimulated cells as compared to control cells, suggesting that the increase of GCLM alone was not sufficient to enhance the levels of GSH in those cells. The second important aspect to stress is that the pre-incubation of intestinal cells with either C3G or Resv, before cytokine-stimulation, was able to significantly increase the expressions of both GCLC and GCLM, as well as the ratio of GSH/GSSG. This contrasted with the pre-incubation of cells with 5-ASA, in which a GCLC and GCLM increase was observed but without a significant increase in the GSH/GSSG ratio. These findings are in harmony with the results obtained by Oz *et al* [41], revealing that green tea polyphenols were more efficient than the drug sulfasalazine, which is a parent compound of 5-ASA, in increasing the levels of colonic GSH in DSS-treated mice.

On the other hand, although both polyphenols and 5-ASA increased Nrf2 pathway in cytokine-stimulated cells, only C3G and Resv [14] were able to significantly decrease the production of reactive species in those cells, demonstrating in some way, the antioxidant superiority of those polyphenols as compared to 5-ASA.

Alongside Nrf2 pathway, PPAR-γ pathway was recently found as important for inhibiting

intestinal inflammatory response and protecting cells from oxidative damage [22, 23, 42]. In general terms, PPAR-γ belongs to a nuclear receptor family of ligand-dependent transcription factors, whose activation requires, at first, its binding to a specific agonist, then its hetero-dimerization with the nuclear retinoid X receptor (RXR) and further the heterodimer binding to sequence-specific regions in DNA, called Peroxisome Proliferator Response Elements (PPRE) [43]. This description corresponds to the conventional mechanism by which PPARs regulate gene expression. However, through a mechanism, not fully understood, termed transrepression, PPAR-γ is able to antagonize the transcriptional activity of several transcription factors, namely NF-kB, STAT1, activator protein-1 (AP-1), without interacting with PPRE [43, 44] and this process has been invoked to clarify the role of PPAR-γ against inflammation [23]. Aiming to determine whether PPAR-γ was involved in the antioxidant and anti-inflammatory protection afforded by C3G and Resv, in comparison with 5-ASA, we evaluated the effects of these polyphenolic compounds on PPAR-γ nuclear accumulation in cells stimulated or not with cytokines. In accordance with some compelling studies evidencing that the anti-inflammatory activity of 5-ASA could be dependent on the activation of PPAR-γ [45], 5-ASA showed to increase, in our experimental conditions, the accumulation of PPAR-γ in the nucleus of cytokine-exposed intestinal cells. Unlike C3G, Resv demonstrated similar results to 5-ASA, at a concentration 20 times lower. Altogether, our results are consistent with the involvement of PPAR-γ pathway in the protection afforded by Resv against inflammation and oxidative stress. This assumption is supported by strong evidence indicating that PPAR-γ can inhibit the transcriptional activation of STAT1, by transrepression, for instance, through direct competition for scarce amounts of co-activator molecules [46]. Remarkably, in our previous work, we demonstrated that, in the same experimental conditions, Resv was able to decrease the amount of activated STAT1 in the nucleus of cytokine-stimulated HT-29 cells [14], being plausible that the remaining activated STAT1 could be transcriptionally inactive in the nucleus of those cells, by Resv-induced PPAR-γ interference. On the other hand, it was found that PPAR-γ may interplay with Nrf2 pathway, inducing Nrf2 activation directly or through upstream pathways [23]. Therefore, it is possible that PPAR-γ activation may have contributed to Resv effect on the enhancement of Nrf2 transcriptional activation in cytokine-stimulated cells.

Based on the above results, our work gives a step forward unravelling Nrf2 and PPAR-γ as signalling pathways involved in the protection afforded by Resv and C3G against cytokine-induced inflammation and oxidative response in HT-29 intestinal cells, making

comparison with the pharmaceutical agent, 5-ASA.

In conclusion, the novel findings that we describe in our work, regarding to the beneficial effects of C3G and Resv against cytokine-induced inflammation and oxidative stress, strengthen the belief that polyphenols abundant in fruits, vegetables and red-wine, typically present in a Mediterranean diet, can be truly advantageous for the prevention of chronic inflammatory diseases, as is the case of IBD, providing even more benefit to patients than that obtained with conventional drugs, such as 5-ASA. However, we are conscious that *in vivo* studies must be conducted to establish evidence for the actual value of dietary polyphenols, as C3G and Resv, in limiting IBD progression, which are planned in a near future.

7. References

1. Hooper, L.V. & Macpherson, A.J. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* **10**, 159-69 (2010).
2. Kamada, N., Seo, S.U., Chen, G.Y. & Nunez, G. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol* **13**, 321-35 (2013).
3. Maynard, C.L., Elson, C.O., Hatton, R.D. & Weaver, C.T. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* **489**, 231-41 (2012).
4. Maloy, K.J. & Powrie, F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* **474**, 298-306 (2011).
5. Peterson, L.W. & Artis, D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol* **14**, 141-53 (2014).
6. Cerf-Bensussan, N. & Gaboriau-Routhiau, V. The immune system and the gut microbiota: friends or foes? *Nat Rev Immunol* **10**, 735-44 (2010).
7. Paiva, C.N. & Bozza, M.T. Are reactive oxygen species always detrimental to pathogens? *Antioxid Redox Signal* **20**, 1000-37 (2014).
8. Abreu, M.T. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol* **10**, 131-44 (2010).
9. Pavlick, K.P. et al. Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. *Free Radic Biol Med* **33**, 311-22 (2002).
10. Biasi, F. et al. Wine consumption and intestinal redox homeostasis. *Redox Biol* **2**, 795-802 (2014).
11. Biasi, F., Leonarduzzi, G., Oteiza, P.I. & Poli, G. Inflammatory bowel disease: mechanisms, redox considerations, and therapeutic targets. *Antioxid Redox Signal* **19**, 1711-47 (2013).
12. Xavier, R.J. & Podolsky, D.K. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **448**, 427-34 (2007).
13. Serra, D., Paixao, J., Nunes, C., Dinis, T.C. & Almeida, L.M. Cyanidin-3-glucoside suppresses cytokine-induced inflammatory response in human intestinal cells: comparison with 5-aminosalicylic acid. *PLoS One* **8**, e73001 (2013).
14. Serra, D., Rufino, A.T., Mendes, A.F., Almeida, L.M. & Dinis, T.C. Resveratrol modulates cytokine-induced Jak/STAT activation more efficiently than 5-aminosalicylic acid: an in vitro approach. *PLoS One* **9**, e109048 (2014).

15. Schneider, M.J. Phytochemicals for the treatment of inflammatory bowel diseases. *Phytochem Rev* **13**, 629-642 (2014).
16. Biasi, F., Astegiano, M., Maina, M., Leonarduzzi, G. & Poli, G. Polyphenol supplementation as a complementary medicinal approach to treating inflammatory bowel disease. *Curr Med Chem* **18**, 4851-65 (2011).
17. Manach, C., Scalbert, A., Morand, C., Remesy, C. & Jimenez, L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**, 727-47 (2004).
18. Romier, B., Schneider, Y.J., Larondelle, Y. & During, A. Dietary polyphenols can modulate the intestinal inflammatory response. *Nutr Rev* **67**, 363-78 (2009).
19. Williamson, G. & Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* **81**, 243S-255S (2005).
20. Kaspar, J.W., Niture, S.K. & Jaiswal, A.K. Nrf2:INrf2 (Keap1) signalling in oxidative stress. *Free Radic Biol Med* **47**, 1304-9 (2009).
21. Kim, J., Cha, Y.N. & Surh, Y.J. A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. *Mutat Res* **690**, 12-23 (2010).
22. Dubuquoy, L. et al. PPARgamma as a new therapeutic target in inflammatory bowel diseases. *Gut* **55**, 1341-9 (2006).
23. Polvani, S., Tarocchi, M. & Galli, A. PPARgamma and Oxidative Stress: Con(beta) Catenating NRF2 and FOXO. *PPAR Res* **2012**, 641087 (2012).
24. Scalbert, A., Manach, C., Morand, C., Remesy, C. & Jimenez, L. Dietary polyphenols and the prevention of diseases. *Crit Rev Food Sci Nutr* **45**, 287-306 (2005).
25. Rousseaux, C. et al. Intestinal antiinflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator-activated receptor-gamma. *J Exp Med* **201**, 1205-15 (2005).
26. Williams, C., Panaccione, R., Ghosh, S. & Rioux, K. Optimizing clinical use of mesalazine (5-aminosalicylic acid) in inflammatory bowel disease. *Therap Adv Gastroenterol* **4**, 237-48 (2011).
27. Hissin, P.J. & Hilf, R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* **74**, 214-26 (1976).
28. Halliwell, B. & Whiteman, M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol* **142**, 231-55 (2004).
29. Brigelius-Flohe, R. & Flohe, L. Basic principles and emerging concepts in the redox

control of transcription factors. *Antioxid Redox Signal* **15**, 2335-81 (2011).

30. Khor, T.O. et al. Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis. *Cancer Res* **66**, 11580-4 (2006).
31. Rushworth, S.A., Shah, S. & MacEwan, D.J. TNF mediates the sustained activation of Nrf2 in human monocytes. *J Immunol* **187**, 702-7 (2011).
32. Speciale, A. et al. Cyanidin-3-O-glucoside counters the response to TNF-alpha of endothelial cells by activating Nrf2 pathway. *Mol Nutr Food Res* **57**, 1979-87 (2013).
33. Hao, E. et al. Resveratrol alleviates endotoxin-induced myocardial toxicity via the Nrf2 transcription factor. *PLoS One* **8**, e69452 (2013).
34. Paine, A., Eiz-Vesper, B., Blasczyk, R. & Immenschuh, S. Signalling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochem Pharmacol* **80**, 1895-903 (2010).
35. Zhu, X., Fan, W.G., Li, D.P., Kung, H. & Lin, M.C. Heme oxygenase-1 system and gastrointestinal inflammation: a short review. *World J Gastroenterol* **17**, 4283-8 (2011).
36. Vijayan, V., Mueller, S., Baumgart-Vogt, E. & Immenschuh, S. Heme oxygenase-1 as a therapeutic target in inflammatory disorders of the gastrointestinal tract. *World J Gastroenterol* **16**, 3112-9 (2010).
37. Wakabayashi, N., Slocum, S.L., Skoko, J.J., Shin, S. & Kensler, T.W. When NRF2 talks, who's listening? *Antioxid Redox Signal* **13**, 1649-63 (2010).
38. Lu, S.C. Glutathione synthesis. *Biochim Biophys Acta* **1830**, 3143-53 (2013).
39. Wu, G., Fang, Y.Z., Yang, S., Lupton, J.R. & Turner, N.D. Glutathione metabolism and its implications for health. *J Nutr* **134**, 489-92 (2004).
40. Franklin, C.C. et al. Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. *Mol Aspects Med* **30**, 86-98 (2009).
41. Oz, H.S., Chen, T. & de Villiers, W.J. Green Tea Polyphenols and Sulfasalazine have Parallel Anti-Inflammatory Properties in Colitis Models. *Front Immunol* **4**, 132 (2013).
42. Annese, V., Rogai, F., Settesoldi, A. & Bagnoli, S. PPARgamma in Inflammatory Bowel Disease. *PPAR Res* **2012**, 620839 (2012).
43. Varga, T., Czimmerer, Z. & Nagy, L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochim Biophys Acta* **1812**, 1007-22 (2011).
44. Ricote, M. & Glass, C.K. PPARs and molecular mechanisms of transrepression. *Biochim Biophys Acta* **1771**, 926-35 (2007).

45. Desreumaux, P. & Ghosh, S. Review article: mode of action and delivery of 5-aminosalicylic acid - new evidence. *Aliment Pharmacol Ther* **24 Suppl 1**, 2-9 (2006).
46. Delerive, P., Fruchart, J.C. & Staels, B. Peroxisome proliferator-activated receptors in inflammation control. *J Endocrinol* **169**, 453-9 (2001).

Chapter 5

Concluding Remarks

Concluding Remarks

The work reported in this thesis highlights the anti-inflammatory potential of two polyphenols typically present in the Mediterranean diet and draws attention to their putative role as adjuvant approach to conventional pharmacological therapy.

The results obtained demonstrated that both the anthocyanin cyanidin-3-glucoside and the stilbene resveratrol were able to efficiently decrease the production of cytokine-induced pro-inflammatory mediators, such as NO and PGE_2, and the expression of cytokine-induced pro-inflammatory enzymes, namely iNOS and COX-2, without affecting the activation of NF-kB, but decreasing the amount of activated STAT1 in the nucleus of human intestinal cells (HT-29). These effects were achieved with concentrations of either cyanidin-3-glucoside or resveratrol twenty times lower than that at which the drug 5-ASA promoted similar effects, reflecting the superior anti-inflammatory efficiency of these natural polyphenolic compounds as compared to the pharmaceutical agent. This study also showed that resveratrol, in contrast to 5-ASA, inhibited the activation of JNK, thus preventing the maximization of the transcriptional potential of STAT1, accumulated in the nucleus of cytokine-stimulated HT-29 cells. Furthermore, neither Resv nor 5-ASA showed to be able to decrease the activation of p38 MAPK in cytokine-stimulated HT-29 cells, which might contribute to the inhibition of the transcriptional activity of the STAT1, in those cells.

On the other hand, considering that inflammation and oxidative stress are crucial close related phenomena in the pathogenesis of IBD, and that Nrf2 signalling pathway has been recognized as the most important cellular defence against oxidative stress, being also relevant in the suppression of the inflammatory process, for instance, by counteracting some pro-inflammatory cascades, as NF-kB pathway, the ability of cyanidin-3-glucoside and resveratrol to induce Nrf2 pathway, in comparison with 5-ASA, was also addressed in the present study. It was demonstrated that cyanidin-3-glucoside and resveratrol were able to induce Nrf2 activation and consequently to increase the ARE-regulated mRNA expression of two important enzymes, HO-1 and GCL. Although the mechanisms behind the protective effects of HO-1 on colitis are not fully understood, some studies indicated that this enzyme is able to inhibit the expression of a crucial pro-inflammatory enzyme, the iNOS, which could be a putative molecular mechanism underlying, at least in part, the beneficial effects of some HO-1 inducers [1, 2]. Strikingly, the increments of HO-1 expression promoted by

cyanidin-3-glucoside and resveratrol in cytokine-stimulated HT-29 cells can hence be related to their ability to inhibit the expression of iNOS and the consequent production of NO in those cells. Moreover, both polyphenolic compounds, as opposed to 5-ASA, were able to enhance the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) and to inhibit the production of reactive species, in cytokine-challenged cells, even at concentrations much lower than those at which 5-ASA was assayed. Alongside Nrf2 pathway, PPAR-γ pathway was recently found as important for inhibiting intestinal inflammatory response and for protecting cells from oxidative damage. Actually, through a process called transrepression, PPAR-γ is able to antagonize the transcriptional activity of several transcription factors, namely NF-kB and STAT1, which might, at least in part, explain the role of PPAR-γ against inflammation. On the other hand, it was found that PPAR-γ may interplay with Nrf2 pathway, inducing Nrf2 activation directly or through upstream pathways. In line with this, resveratrol was found in the present work to increase the amount of PPAR-γ in the nucleus of cytokine-stimulated HT-29 cells. Therefore, it is proposed in the present work a potential involvement of PPAR-γ in the ability of resveratrol to counteract STAT1 activation and to enhance Nrf2 transcriptional activation in cytokine-stimulated HT-29 cells. Although 5-ASA showed similar effects to those of resveratrol, they were obtained at a 20 times higher concentration of 5-ASA.

Moreover, it is worth noting that, although the low oral bioavailability of the majority of polyphenols can be considered a handicap for its therapeutic employment [3], this does not constitute a true problem in the particular case of IBD, since several studies [3-5] have indicated that the intestine is the organ where dietary polyphenols achieve the highest concentrations in the human body (up to several hundred μM). Therefore, the selected concentration for the polyphenols under this study, 25 μM, could be easily reached in the gut lumen, after consumption of polyphenol-rich fruits or beverages. On the other hand, the concentration of 5-ASA, 500 μM, was selected taking into consideration the levels reached by this drug in colonic mucosa [6].

Altogether, the results of the present work testify the anti-inflammatory and the antioxidant properties of two structurally different polyphenolic compounds, one flavonoid, the anthocyanin cyanidin-3-glucoside, and one nonflavonoid, the stilbene resveratrol, both present in the Mediterranean diet, thus strengthening the belief that this diet, typically enriched in polyphenols abundant in fruits, vegetables and red-wine, can be truly advantageous for minimizing the periods of relapses and for limiting the progression of IBD, providing even

more potential benefit to patients than that obtained with conventional drugs, such as 5-ASA. On the other hand, by shedding light on the cellular signalling pathways through which those polyphenols exert their effects against inflammation and oxidative stress, these studies pave the way for the discovery of other compounds or for the structural refinement of pre-existing ones that, through modulating the expression and activation of some of the proteins involved in key signalling pathways, can be envisaged as useful "weapons" against intestinal inflammation and oxidative stress, typically present in IBD patients.

Although in this study there is no evidence to support an additive or synergistic effect between cyanidin-3-glucoside or resveratrol and 5-ASA, this effect cannot be excluded in animal models of the disease or in human patients, since, as referred in the Introduction section, immune cells, such as dendritic cells, also play a crucial role in the pathogenesis of IBD and are not taken into account in this *in vitro* study. Therefore, we are aware that *in vivo* studies must be undertaken to establish evidence for the actual value of dietary polyphenols, as cyanidin-3-glucoside and resveratrol, in the context of IBD, either alone or in combination with the drug 5-ASA.

References

1. Zhu, X., Fan, W.G., Li, D.P., Kung, H. & Lin, M.C. Heme oxygenase-1 system and gastrointestinal inflammation: a short review. *World J Gastroenterol* **17**, 4283-8 (2011).
2. Wakabayashi, N., Slocum, S.L., Skoko, J.J., Shin, S. & Kensler, T.W. When NRF2 talks, who's listening? *Antioxid Redox Signal* **13**, 1649-63 (2010).
3. Williamson, G. & Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* **81**, 243S-255S (2005).
4. Biasi, F. et al. Wine consumption and intestinal redox homeostasis. *Redox Biol* **2**, 795-802 (2014).
5. Romier, B., Schneider, Y.J., Larondelle, Y. & During, A. Dietary polyphenols can modulate the intestinal inflammatory response. *Nutr Rev* **67**, 363-78 (2009).
6. Williams, C., Panaccione, R., Ghosh, S. & Rioux, K. Optimizing clinical use of mesalazine (5-aminosalicylic acid) in inflammatory bowel disease. *Therap Adv Gastroenterol* **4**, 237-48 (2011).

Appendix

Copyright License Agreements

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

Sep 22, 2015

This is a License Agreement between Diana Serra ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3714260906730
License date	Sep 22, 2015
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Regional specialization within the intestinal immune system
Licensed content author	Allan M. Mowat, William W. Agace
Licensed content date	Sep 19, 2014
Volume number	14
Issue number	10
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Modulation of intestinal inflammation by dietary polyphenols in comparison with 5-aminosalicylic acid: an in vitro approach
Expected completion date	Sep 2015
Estimated size (number of pages)	175
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested

indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

   Note: For republication from the *British Journal of Cancer,* the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   Note: For adaptation from the *British Journal of Cancer,* the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information.Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

   Note: For translation from the *British Journal of Cancer,* the following credit line applies.
   Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.
Special Terms:

v1.1

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Sep 22, 2015

This is a License Agreement between Diana Serra ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3714261434636
License date	Sep 22, 2015
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Immune adaptations that maintain homeostasis with the intestinal microbiota
Licensed content author	Lora V. Hooper and Andrew J. Macpherson
Licensed content date	Mar 1, 2010
Volume number	10
Issue number	3
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Modulation of intestinal inflammation by dietary polyphenols in comparison with 5-aminosalicylic acid: an in vitro approach
Expected completion date	Sep 2015
Estimated size (number of pages)	175
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in

the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

   Note: For republication from the *British Journal of Cancer,* the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   Note: For adaptation from the *British Journal of Cancer,* the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information.Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

   Note: For translation from the *British Journal of Cancer,* the following credit line applies.
   Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Special Terms:
v1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

Sep 22, 2015

This is a License Agreement between Diana Serra ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3714270163040
License date	Sep 22, 2015
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Anatomical basis of tolerance and immunity to intestinal antigens
Licensed content author	Allan McI. Mowat
Licensed content date	Apr 1, 2003
Volume number	3
Issue number	4
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Modulation of intestinal inflammation by dietary polyphenols in comparison with 5-aminosalicylic acid: an in vitro approach
Expected completion date	Sep 2015
Estimated size (number of pages)	175
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested

indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

   Note: For republication from the *British Journal of Cancer,* the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   Note: For adaptation from the *British Journal of Cancer,* the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information.Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

   Note: For translation from the *British Journal of Cancer,* the following credit line applies.
   Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.
Special Terms:

v1.1

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Sep 22, 2015

This is a License Agreement between Diana Serra ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3714270384850
License date	Sep 22, 2015
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Acquired and natural memory T cells join forces at the mucosal front line
Licensed content author	Hilde Cheroutre and Loui Madakamutil
Licensed content date	Apr 1, 2004
Volume number	4
Issue number	4
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 2
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Modulation of intestinal inflammation by dietary polyphenols in comparison with 5-aminosalicylic acid: an in vitro approach
Expected completion date	Sep 2015
Estimated size (number of pages)	175
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in

the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

   Note: For republication from the *British Journal of Cancer,* the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   Note: For adaptation from the *British Journal of Cancer,* the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information.Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

   Note: For translation from the *British Journal of Cancer,* the following credit line applies.
   Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Special Terms:
v1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Sep 22, 2015

This is a License Agreement between Diana Serra ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3714270557878
License date	Sep 22, 2015
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Intestinal epithelial cells: regulators of barrier function and immune homeostasis
Licensed content author	Lance W. Peterson, David Artis
Licensed content date	Feb 25, 2014
Volume number	14
Issue number	3
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 3
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Modulation of intestinal inflammation by dietary polyphenols in comparison with 5-aminosalicylic acid: an in vitro approach
Expected completion date	Sep 2015
Estimated size (number of pages)	175
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in

the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

   Note: For republication from the *British Journal of Cancer,* the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   Note: For adaptation from the *British Journal of Cancer,* the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information.Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

   Note: For translation from the *British Journal of Cancer,* the following credit line applies.
   Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Special Terms:
v1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.