博士論文 （要約）

Effects of apple-derived condensed tannins and pomegranate-derived polyphenols on cytokine expression of intestinal epithelial cells

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PREFACE

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Abbreviations:

ACT: apple condensed tannin

BSA: bovine serum albumin

CD: Crohn's disease

CXCL: C-X-C motif ligand

DMEM: Dulbucco's Modified Eagle Medium

DMSO: dimethyl sulfoxide

ELISA: enzyme-linked immunosorbent assay

ERK: extracellular signal-regulated kinase

HPRT: hypoxanthine phosphoribosyltransferase

HPLC: high pressure liquid chromatography

IBD: inflammatory bowel diseases

IEC: intestinal epithelial cell

IEL: intraepithelial lymphocyte

IFN: interferon

IκB: Ikappa B

IL: interleukin

IRF3: interferon regulatory factor 3

JNK: c-jun N-terminal kinase

LDH: lactate dehydrogenase

LPS ： lipopolysaccharide

LY: Lucifer Yellow

MAPK: mitogen-activated protein kinase

MEK: MAPK/ERK kinase

NF-κB: nuclear factor kappa B.

NK: natural killer

O.D.: optical density

PBS: phosphate buffered saline

Poly I:C: polyinosinic-polycytidylic acid

PI3K: phosphatidylinositol 3-kinse

PKC: protein kinase C

PAE: pomegranate aril extract

PPE: pomegranate peel extract

PLE: pomegranate leaf extract

qRT-PCR: quantitative reverse transcription-polymerase chain reaction

STAT: signal transducer and activator of transcription.

TLR: Toll-like receptor

TGF: transforming growth factor

TNF: tumor necrosis factor

UC: ulcerative colitis

Chapter 1. General Introduction

1. General Introduction

The primary function of the gastrointestinal tract is digestion, absorption, and assimilation of nutrients. The gastrointestinal tract has the largest surface area among all organs. In fact, the 400 square meter surface area of the gastrointestinal tract is about 200 times larger than that of the entire skin. Moreover, it is estimated that lymphocytes in intestinal immune system may account for more than 60% of the total body lymphocytes (see Figure 1-1). Inflammatory Bowel Disease (IBD) is chronic inflammatory disease of the gastrointestinal tract. IBD has traditionally been divided into Crohn's disease (CD) and ulcerative colitis (UC). Crohn's disease and Ulcerative Colitis are differentiated by their clinical manifestations and hypothesized pathogenic mechanisms. UC is relapsing non-transmural chronic inflammatory disease that is restricted to the colon and during disease flares characterized by bloody diarrhea [1]. Crohn's disease is a chronic, segmental localized granulomatous disease that can affect anywhere in the entire gastrointestinal tract from the mouth to anus. Whereas ulcerative colitis only involves the innermost layer of the colon, CD causes chronic pain and inflammation throughout the gastrointestinal tract, causing swelling, constipation, persistent, diarrhea, rectal bleeding, severe abdominal pain, and cramping. The symptoms of UC are the same as CD but also include fever, decreased appetite, fatigue, and unhealthy weight loss. CD and UC can appear at any age, but most patients are diagnosed in their third decade of life [2].

The etiology of IBD is still unknown but studies indicate several possible causes such as genetics [3], immunology [4, 5], nutrition [6], bacteria [7], viruses [8, 9], and other environment factors. Cytokines play a key role in the intestinal immune system, and are known to participate in the disruption of the so-called normal state of controlled inflammation (see Figure 1-2). Cytokines are released mainly by immune cells that facilitate communication between cells and mediate the systemic inflammation. Several immune cells secrete cytokines that actively regulate the inflammatory response in IBD. Once secreted by these antigen presenting cells, these cytokines trigger and differentiate T cells activating. Accumulated animal model studies suggest that inflammation in IBD patients likely occurs as a result of either excessive effector T-cell function or deficient regulatory T-cell function, associated with overproduction of pro-inflammatory cytokines, such as TNF-α, IL-12, or deficiency in the production or function of known immunosuppressive cytokines such as IL-10 [7, 10]. High serum levels of TNF-α expression in both patients with CD and UC were measured, this suggest that the levels of TNF-α correlate with clinical and laboratory indices of intestinal disease activity. [11] In addition, clinical studies have reported a dramatic improvement in CD patients treated with anti-TNF-α therapy such as infliximab and adalimumab [12].

In contrast to other cytokines, IL-6 is a pleiotropic cytokine that exerts its proinflammatory effects largely by means of its soluble IL-6 receptor (sIL-6R). Accumulated studies suggested that altered IL-6 production has been found in chronic inflammatory bowel disease (IBDs) affected patients [13-15]. It is well-known that IL-6, together with TGF-β, induces the activation, proliferation, and differentiation of naïve T cells into Th17 T cells, leading to increased inflammatory cytokines, such as IL-22 and IL-17 that induce intestinal bowel disease (see Figure 1-2). IL-6 signaling through signal transducer and activator of transcription-3 (STAT3) has been extensively studied. This system plays a central role in circulating levels of IL-6 and sIL-6R, resulting in activation of several immunologic reactions during the development of IBD [16, 17]. A very interesting report indicated that the treatment with anti-IL-6 receptor monoclonal antibody reduced IFN-γ, TNF-α, and IL-1β mRNA, and also suppressed the expression of several intracellular adhesion molecules in the colonic vascular endothelium [18]. In addition, it has also been reported that blockade of IL-6 trans-signaling causes T-cell apoptosis, indicating that the IL-6-sIL-6R system mediates the resistance of T cells to apoptosis in CD [19].

IL-6 is produced by various cell types, such as monocytes [20], B cells [21], endothelial cells [22, 23] , fibroblasts [24], and epithelial cells [25][26], and IL-6 production is increased in various conditions characterized by inflammation such as sepsis, endotoxemia, and treatment with pro-inflammatory cytokines, such as IL-1β [27, 28], and Toll-like receptor ligands, Pam3CSK4, Poly I:C, and LPS [29, 30]. The transcriptional regulation of the IL-6 gene is complex and involves at least four different transcription factors, including activator protein (AP)-1, NF-κB, CCAAT/enhancer binding protein (C/EBP), and cAMP response element (CRE)-binding protein (CREB) [31]. The role of these transcription factors varies between different cell types and may also vary different signaling pathways.

Yong-Hae Son et al, has reported that enhanced release of IL-6 was connected with high activation of MAPK and NF-κB signaling pathways [32]. It has also been reported that IL-1β stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF-κB [33]. In addition, hepatitis C virus (HCV) core protein activates MAPK signaling pathways, ERK and p38 MAPK as well as IκB in the liver of core-transgenic mice fed with short-term, ethanol-containing diets [34]. Another study showed that LPS induced IL-6 through transcriptional activation via activating ERK1/2, p38 MAPK and NF-κB pathway [32]. Furthermore, several reports demonstrated that natural products down-regulated NF-κB activation and inhibited the phosphorylation of IκB kinase complex

(IKK) [35, 36] [37]. Taken together, previous studies suggest that MAPKs signaling pathway and IκB signaling pathway play an important role in the expression of IL-6 gene.

Chemokines are a family of small (8-10 kDa) protein, are classified into constitutively secreted and inducible. Chemokines play a crucial role in mucosal immunity and inflammation via recruitment and activation of leukocytes. Especially, inducible chemokines are inflammatory molecules responsible for mediating the recruitment of leucocyte effector populations to the sites of immune reaction [38]. It has been reported that some chemokines are important modulators of monocyte-endothelial interactions under flow conditions [39]. A previous report also suggest that chemokines have been implicated in the pathogenesis of airway inflammation in asthma [40]. Although the etiology of inflammatory bowel disease (IBDs) is not very clear, in the past few years accumulated evidence indicated that chemokines play a central role in the pathogenesis of both forms of IBD [41, 42].

Several chemokines have been investigated in both CD and UC, and their expression is consistently increased during the active phases of the disease. Recruitment of activated T-cells to the inflammation sites is a common feature in a wide variety of inflammatory bowel disease. CXCL10 and its receptor are up-regulated in the colonic mucosa [43], they may have a role in activated T lymphocytes recruitment into the intestinal mucosa. Three ligands CXCL9 (Mig), CXCL10 (IP-10), and CXCL11 (I-TAC) are known to activate a common receptor CXCR3. Among them, CXCL10 and CXCL11 are the most potent inducer of CXCR3 internalization and are the physiologic inducer of CXCR3 internalization after T cell contact with activated endothelial cells. CXCR3 is expressed on the surface of a number of cell types, including activated T cells, B cells, and NK cells, and subsets of inflammatory dendritic cells, macrophages [44, 45] (see Figure 1-1). Additionally, another paper also indicate that CXCL10 selectively attracts activated T lymphocytes, which are the only inflammatory cells expressing the chemokine receptor CXCR3 [44]. Recently, it also has been reported that up-regulated chemokines CXCL10 lead to enhanced cancer cell death by driving tumor infiltration by T and NK cells [46]. These findings suggest that CXCL10 play important role in inflammatory bowel disease (see Figure 1-2).

These chemokines are induced by several different stimulations and are produced in different cell types. Accumulated evidence suggest that CXCL9, CXCL10, and CXCL11 are released from both airway epithelial cells and airway smooth muscles cells, can be potentiated by the synergistic interaction of TNF-α with IFN-γ [47, 48]. In addition, Peter S. et al. has shown that the human airway epithelial cell line BEAS-2B could release CXCL9, CXCL10, and

CXCL11 in responsive to IFN-γ via activation of IκB kinase, IKK2, but not activation of NF-κB [49]. CXCL10 may be induced by hepatitis C virus (HCV) within hepatocytes but not by other cell types within the liver [50]. Jason C.L. et al., have reported that human rhinovirus (HRV) infections strikingly increased CXCL10 production in nasal lavages *in vivo*. In addition, they found that CXCL10 expression was robustly increased in human airway epithelial cells induced with synthetic double-stranded RNA, however single-stranded RNA had no effect [51].

High levels of CXCL10 release have been found in patients with UC or CD and intestinal epithelial cell (IEC) lines. A report suggests that the gene expression of CXCL10 and its receptor CXCR3 are up-regulated in colonic mucosa in active IBD [52]. Laura J. et al. reported that in the large intestine, the chemokine CXCL10 and cytokine interleukin-13 regulate the intestinal epithelial cell movement [53]. Other previous reports have also shown that chemokines CXCL9, CXCL10, and CXCL11 can be produced by intestinal epithelial cells and induced by IFN-γ treatment [54] [55].

Toll-like receptor 3 (TLR3), a member of the TLR family proteins, is localized to the endosomal compartment of some types of cells. TLR3 recognizes viral double-stranded RNA and its synthetic analog polyribonosinic: polyribocytidylic acid (Poly I:C) [56]. Upon recognition of Poly I:C or dsRNA virus, TLR3 transmits signals via the adaptor protein Toll-IL-1 receptor (TIR), to activate the transcription factors NF-ϰB, AP-1, and IRF3. Activation of these signaling pathways leads to the induction of cytokine and chemokines productions [57]. Numerous previous studies have shown that among the TLR family members, only TLR3 does not activate the downstream signaling pathways by using myeloid differentiation factor 88 (MyD88). In contrast, another report suggests that Myd88 is involved in dsRNA-induced cellular responses in MyD88-deficient (MyD88-/-) mice or MyD88-/- macrophages [58].

As shown in Figure 1-3, TLR3 is expressed in the endosomal compartments and recognizes extracellular viral dsRNA and its synthetic analog Poly I:C. Once, TLR3 is dimerized by internalized dsRNA, it recruits the adaptor protein TICAM-1/TRIF. TRAF6-TICAM-1 is oligomerized, inducing the activation of their downstream signaling pathways, including NF-ϰB signaling pathway and MAPKs signaling pathway. Poly I:C shows the same effect on TLR3, involving in the TLR3-TICAM-1-mediated IRF3 activation [58]. Previous findings reported by several groups have shown that Poly I:C/dsRNA treatment induces activation of TLR3-TICAM-1, which resulted in NF-ϰB activation and apoptosis [59-61]. The relevance of MEK1/2-, JNK-, and p38-MAPKs signaling in several cell types has already been demonstrated in the regulation of activator protein1 (AP-1) [62]. TLR3-mediated

signaling events has also been implicated in MEK1/2-, JNK-, and p38-MAPKs signaling pathway. Additionally, it has been reported that activation of TLR4 by LPS and the activation of TLR1/2 by Pam3CSK4 could enhance MAPK kinase1/2 and JNK signaling pathway. Hence, TLR3-TICAM-1-mediated signaling events are involved in NF-κB signaling pathway and MAPKs signaling pathways, and the activation of IRF3.

Over the past three decades, epidemiological studies have shown that diets rich in fruits and vegetables have been associated with reduced risk of developing chronic diseases, such as diabetes, Alzheimer's disease, cardiovascular disease, and certain types of cancers [63-65]. Fruits and vegetables have also been shown to have potent anti-oxidant and anti-inflammatory activities. It is also reported that an imbalance in consumption of vegetables and fruits is associated with increased risk for inflammatory bowel diseases (IBDs). This suggests that increasing the consumption of vegetables and fruits is a practical strategy for significantly reducing the risk of chronic diseases.

Apples (*Malus pumila*) are one of the commonly consumed fruit in many countries of the world for centuries. They are eaten both raw and in processed products such as juice, cider, brandy, jam and vinegar. Apart from its nutritious value, various scientific researches are focused on to investigate the ability to utilize apple for prevention of various degenerative diseases. "An apple a day keeps the doctor away" is still quite popular. Recently, accumulated studies have provided the scientific backing for this very common phrase. Apple contains many types of phenolic substances, such as epicatechin, catechin, flavonoids, and procyanidins (see Figure 2-1) [66, 67]. Among these compounds, apple condensed tannins (ACT) are main contained in apple peels, especially in unripe apple peels. While ACT consist of oligomeric (-)-epicatechin of 2-14 mer, and are comprise over 75.6% of total polyphenols in apple peel extracts (see Table 2-1) [68]. Most importantly, many of these phytochemicals from apple have been widely studied, and those health benefits of apple and apple products have been positively associated with apple polyphenols.

Accumulated evidence indicates that the consumption of apple and apple products could reduce risks of cardiovascular diseases, cancers, asthma, diabetes, obesity, and pulmonary dysfunction [69]. Apple polyphenols have been found to have antioxidant activity [70-73]. Additionally, it has been reported that apple peels have higher antioxidant activity and higher bioactivity than the apple flesh. Apples without the peels had less antioxidant activity than apples with the peels. Apple polyphenols have also been shown to have strong anticancer [74-76], and anti-inflammatory activity[73, 76, 77].

Pomegranate (*Punica granatum* L.) is a very common fruit in Iran, Indian subcontinent, and the Mediterranean countries such as Turkey, Egypt, Tunisia, Spain, and Morocco, is consumed fresh and in processed forms as wine and juice [78]. The biological activity of pomegranate fruits has been widely investigated, including *in vitro, in vivo,* and clinical studies. Since the phytochemicals components of different part of pomegranate fruit are different, the bioactivities of each part of pomegranate fruit are different. Most importantly, numerous scientific evidences proved that the biological activities of pomegranate juice, pomegranate peel,and pomegranate leaf extracts have been associated with their phenolic composition.

Identification and analysis of bioactive components of pomegranate juice show that pomegranate juice contains more than 100 different phytochemicals, primarily including phenolic compounds such as gallic acid, chlorogenic acid, caffeic acid, catechin, quercetin, rutin, and minerals. It also largely contains tannins, mainly ellagitannins and gallotannins. Punicalagins and punicalin are two ellagitannins monomer, are unique to pomegranate fruits (see Figure 4-1 A and B) [79]. The bioactivity of pomegranate juice in general is largely attributed to the presence of these compounds [80]. Accumulated epidemiological studies suggest that continuous consumption of pomegranate juice has been associated with reduced risk of a number of chronic diseases such as cardiovascular diseases (CVD) [81, 82]. Pomegranate juice has also been shown to have potent antioxidant capacity compared to green tea, red wine and fruit juices such as grape, cranberry, and orange juice [83] [84] [85].

Pomegranate peel extract (PPE) has been used as traditional medicine to cure intestinal disease by different cultures. In Egyptian culture, Indian culture and Chinese culture, several common ailments such as intestinal inflammation, diarrhea, and intestinal worms have been treated by exploiting PPE. Identification and analysis of bioactive components of pomegranate peels revealed that pomegranate peel mainly contains ellagitannins. Ellagitannin monomer punicalagin is the largest and most important components in PPE, and is unique to pomegranate arils and peels (see Figure 4-1 C) [86]. More importantly, investigation of pomegranate phytochemicals showed that health benefits of pomegranate peels had been positively associated with the ellagitannins and other polyphenols. Accumulated evidence proves that pomegranate peel has many health benefits, including anti-oxidant, anti-cancer, anti-mutagenic properties. Among those bioactivities, PPE has been shown to have the most potent anti-inflammatory activity, because it may inhibit pro-inflammatory cytokines expression [87].

Pomegranate leaves were also used as a traditional medicine from ancient. In China,

pomegranate leaves have been developed into green tea. Pomegranate leaf extract (PLE) has shown numerous pharmacological activities including antidiarrheal, anti-obesity, anti-bacterial, astringent, and antitumor activity [88, 89]. PLE has also been found to have free radical scavenging activity and antioxidant effects *in vitro* [90]. PLE has different compositions from pomegranate aril and peel extracts. Pomegranate leaf contains ellagic acid and fatty acids. Phytochemical studies previously have revealed strictinin is the main components present in pomegranate leaves (Figure 4-1 D). Importantly, these pharmacological activities from pomegranate leaf have been shown to be associated with flavone glycosides and gallo- and ellagitannins in leaves [91].

However, the effect of ACT on cytokines expression of intestinal epithelial cells is still not very clear. In addition, no reports have directly compared the effects of pomegranate aril extract (PAE), PPE, and PLE on cytokines expression of intestinal epithelial cells. In the present study, apple condensed tannin-induced and pomegranate extracts-induced IL-6, CXCL10 expression was analyzed in intestinal epithelial cells. In addition, the expression and phosphorylation of the signal transduction proteins MEK, ERK, JNK, IκB, and p65 were investigated by western blot analysis. Furthermore, functional inhibitors of the molecules involved in the signaling pathways were used to reveal the mechanism.

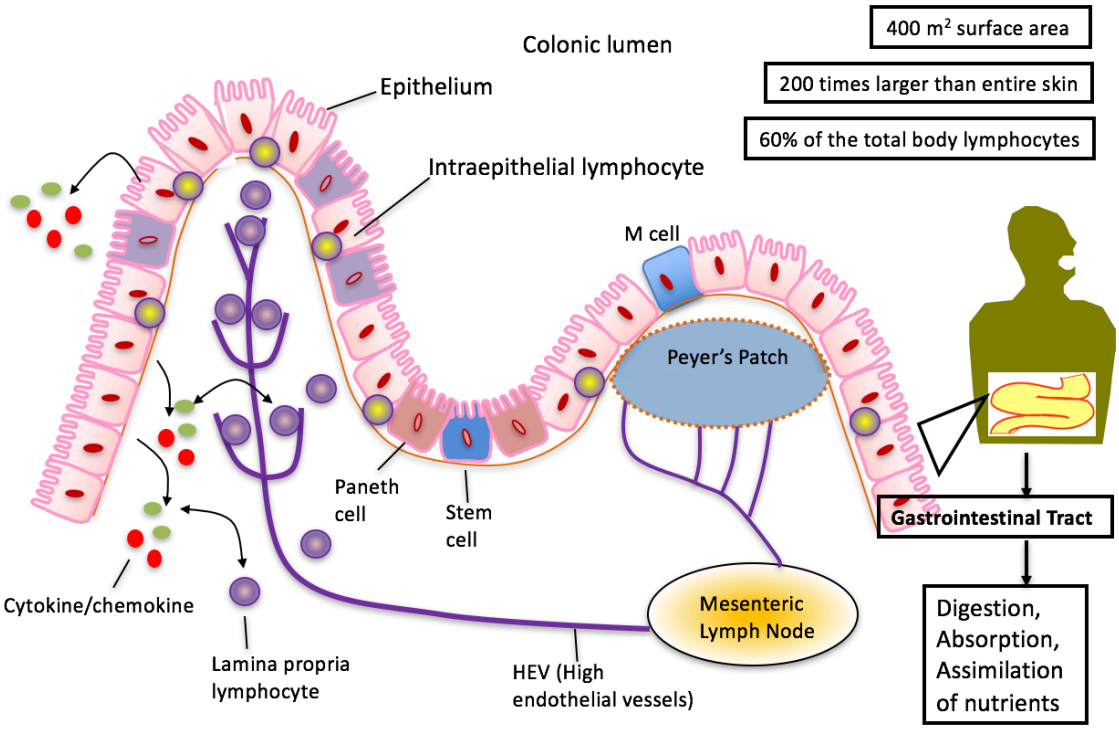


Figure 1-1 Organization of intestinal immune system

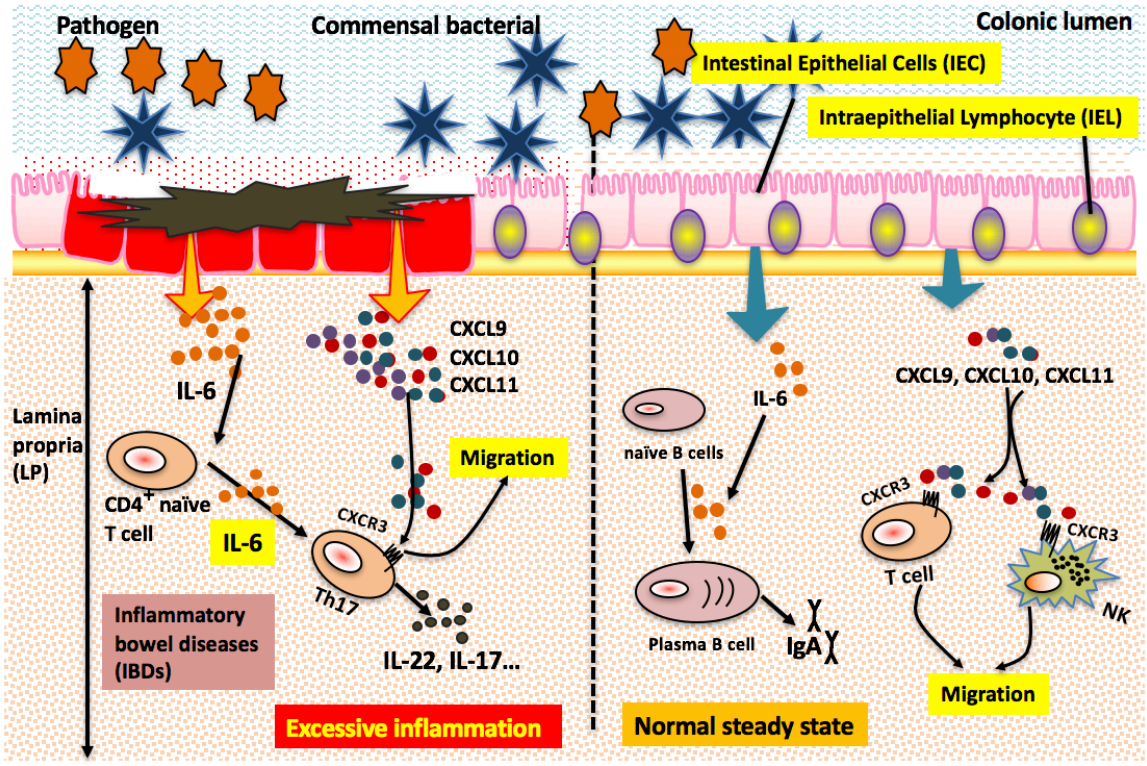


Figure 1-2 The role of IL-6, CXCL9, CXCL10, and CXCL11 in intestinal immune system in inflammation and steady state

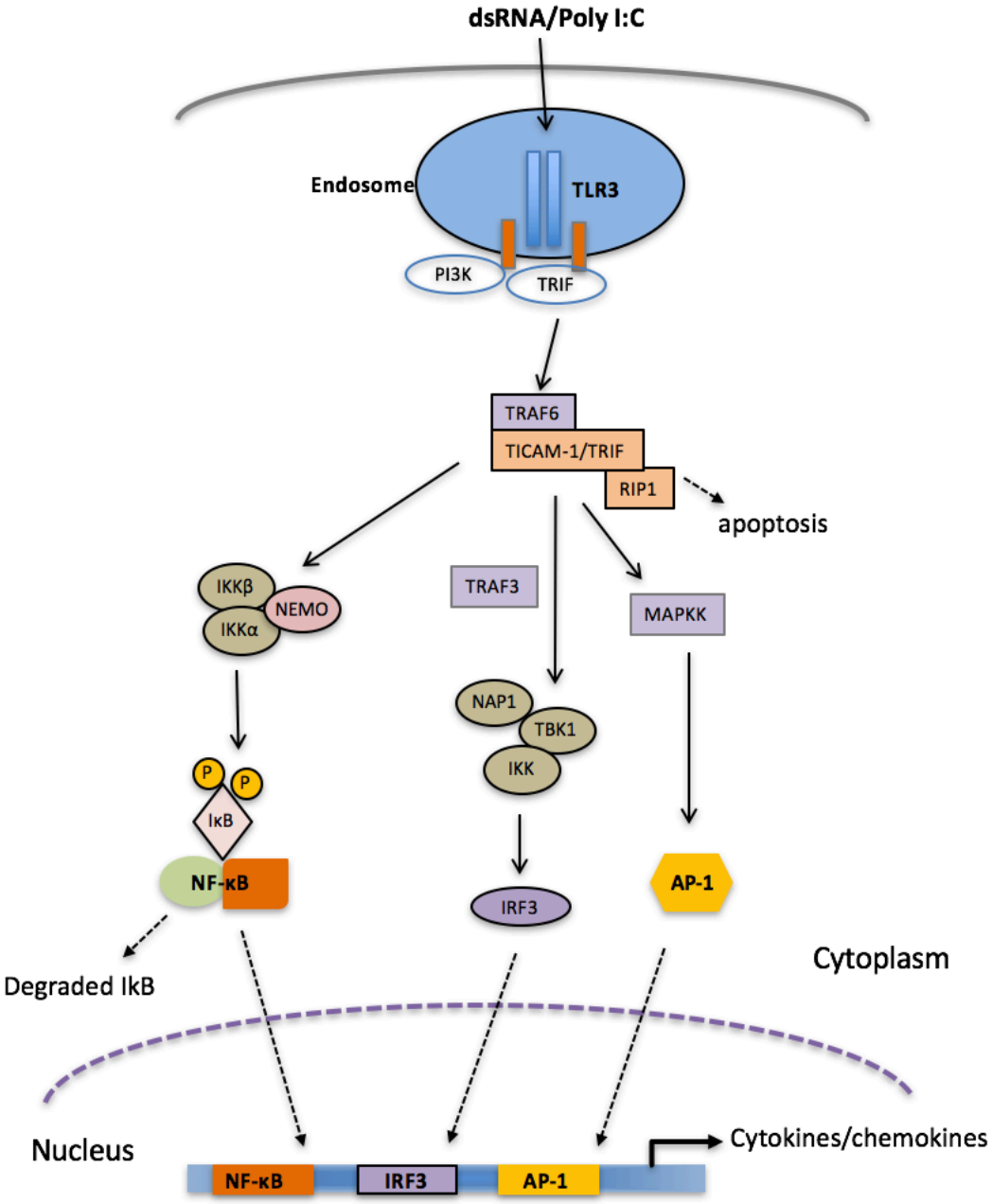


Figure 1-3 Poly I:C-TLR3-mediated signaling pathway

Chapter 2. Polymerization degree-dependent inhibitory effect of apple condensed tannin on interleukin-6 production by intestinal epithelial cells

本章の内容は、これから学術誌に投稿する計画かがあるため、公表できない。2017 年以内投稿する予定。

Chapter 3. Apple condensed tannin regulate CXCL9, CXCL10, and CXCL11 expression via activation of MAPKs signaling pathway in intestinal epithelial cells

本章の内容は、これから学術誌に投稿する計画かがあるため、公表できない。2017 年以内投稿する予定。

Chapter 4. Pomegranate peel-derived polyphenols stimulates CXCL10 expression via activation of PKC, MEK1/2, ERK1/2, JNK and PI3K-IRF3 signaling pathways in intestinal epithelial cells

本章の内容は、これから学術誌に投稿する計画かがあるため、公表できない。2017 年以内投稿する予定。

Chapter 5. General Discussion and Perspectives

The gastrointestinal tract is the largest mucosal surface in our body and is also in continuous contact with dietary antigens and diverse microorganisms. Thus, intestine mucosa surfaces play very importantly, and crucial role in innate and adaptive immune regulation. In the intestinal immune, the epithelial cells establish and maintain the barrier. In addition to that, the epithelial cells release various cytokines and chemokines, such as TNF, IFN-γ, IL-6, IL-8, CXCL10 to regulate the intestinal functions: to protect the mucous membranes against invasion by potentially dangerous microbes, and to prevent the development of potentially harmful immune responses. In addition, intestinal lamina propria and intraepithelial lymphocytes express various receptors to bind these cytokines or chemokines. These cytokines and chemokines are rapidly synthesized and secreted by epithelial cells and intraepithelial lymphocytes upon stimulation and induce the production of adhesion molecules and other inflammatory mediators such as nitric oxide (NO), reactive oxygen metabolites, and PGE.

The role of cytokine and chemokine in the intestinal mucosal immune system has been studied a lot. In inflammatory bowel disease, evidence has been found for a disturbed balance between pro-inflammatory and anti-inflammatory cytokines. Accumulated studies suggested that higher IL-6 production has been found in chronic inflammatory bowel disease affected patients [12-15]. IL-6 is produced by various cell types, including monocytes, endothelial cells, fibroblasts, and epithelial cells, when the cells are treated with different stimulations and are in various inflammatory conditions such as sepsis, endotoxemia, [27-30].

Some reports suggest that activation of TLR1/2, TLR3, and TLR4 pathways involved in the enhancement of IL-6 production [111], [112]. In the present study, I investigated the effect of apple condensed tannin (ACT) on Poly I:C-TLR3-induced IL-6 expression in intestinal epithelial cells. I found that ACT significantly inhibits Poly I:C-TLR3 mediated IL-6 expression in mouse intestinal epithelial cells.

On the transcriptional level, the inducible IL-6 expression is regulated by the transcription factors C/EBP, NF-κB, and CREB [105]. It has been shown that increased release of IL-6 was connected with elevated activation of MAPKs and NF-κB signaling pathways. It has been reported that IL-1β stimulation induced IL-6 production through activation of MAP kinase signaling pathway and NF-κB [33]. Accumulated studies suggest that virus dsRNA selectively activates MEK, ERK, JNK and p38 MAPK signaling pathways, as well as IκB in airway epithelial cells and intestinal epithelial cells. These above evidence prove that MAPKs

signaling pathway and IκB signaling pathway play a crucial role in the expression of IL-6 gene. Here, I hypothesized that ACT inhibits IL-6 expression via suppressing MAPKs and NF-κB signaling pathways. To elucidate the inhibitory effect of ACT on the IL-6 expression, I assessed ACT modulation of the phosphorylation of MEK1/2, JNK, P38 and IκB. The results suggest that ACT significantly inhibits Poly I:C-induced MEK1/2, JNK (p46), p38 phosphorylation and IκB phosphorylation. In addition to my results, accumulated studies have also suggested that natural products could inhibit the phosphorylation of IκB kinase complex (IKK) [36, 37, 115, 182].

As shown in Table 1-1, ACT is the mixture of condensed tannins, which consists of oligomeric (-)-epicatechin of 2-14 mer. To identify the constituents responsible for the down-regulation of IL-6 gene expression, I studied the inhibitory effect of ACT, smaller fractions representative ACT (2-mer) and larger fractions representative ACT (7-mer) on IL-6 gene expression, and IL-6 production, and gene transcription. I found that ACT and ACT (7-mer) inhibit Poly I:C-induced IL-6 gene expression and production. Interestingly, the ACT (7-mer) showed the more powerfully inhibitory effect on IL-6 expression (Figure 2-6 A and B). In addition, I evaluated the effect of each fraction on IL-6 promoter activities. As shown in Figure 2-7, ACT and ACT (7-mer) significantly inhibit IL-6 gene transcriptional activity as well. In contrast, the smaller fractions representative ACT (2-mer) do not effect on IL-6 gene expression, production and gene transcription. Furthermore, I compared the inhibitory effect of ACT, ACT (7-mer) and ACT (2-mer) on the phosphorylation of MEK1/2, JNK, p38, and IκB at the 45 min time point. The results suggest that ACT (7-mer) markedly suppress the phosphorylation of IκB and MEK1/2, JNK, and p38, whereas, ACT (2-mer) did not inhibit the phosphorylation of these four molecules (Figure 2-9). As shown in Figure 2-8, I found that ACT may robustly inhibits the phosphorylation of MEK1/2, JNK and p38 at the time point of 90 min. However, ACT inhibits the phosphorylation of IκB in the early stage than MEK1/2, JNK and p38. Taken together, in Chapter 2, I demonstrate that ACT and lager fraction ACT (7-mer) inhibit Poly I:C-induced IL-6 expression via suppressing the phosphorylation of MEK1/2, JNK (p46), p38, and IκB.

It is well known that tannin could combine with protein to form haze (turbidity) to influence physical properties. Much work has been done over many years to confirm the interactions of condensed tannins and proteins. It has been suggested that condensed tannin-protein interactions are similar to antigen-antibody interactions in that a binding agent and ligand of comparable sizes associate multivalently to form soluble and insoluble complexes.

Hydrogen bonding between phenolic hydroxyl and peptide carbonyl is the major force stabilizing condensed tannin-protein complexes.

It has been shown that the protein-binding affinity of condensed tannins is different. In general, the biological activities of condensed tannins are recognized to be largely dependent on their molecular weight and structure [183]. Condensed tannins of higher molecular weight fractions have stronger protein-binding affinity than those of lower molecular weights. In contrast, protein dose not bind with smaller fraction procyanidin such as dimeric, and trimeric procyanidin. In addition, a couple of studies suggest that relative affinities of proteins and condensed tannins dependent on the size of the protein polymer. The affinities of low molecular weight protein for condensed tannins are at least a thousand-fold lower than those of proteins and large polymers [117]. The hydroxyl groups are excellent at forming hydrogen bonds with proteins. A common characteristic of proteins with high affinity for tannin is their high proline residues. Hydrogen bonding between phenolic hydroxyl and proline residues in the protein is the major force stabilizing condensed tannin-protein complexes. A larger fraction of condensed tannin has a large number of hydroxyl groups, and it consists of many large hydrophobic rings. In addition, the capacity of condensed tannins to bind proteins is a function of the polymer chain length-the larger the condensed tannin, the greater the binding ability. (see Figure 5-1).

Furthermore, condensed tannins have also been found to exhibit protein-binding ability and could protect proteins from being degraded in rumen by forming condensed tannin-protein complexes. The condensed tannin-protein complexes are then hydrolyzed in the small intestine releasing the proteins for digestion and absorption [184]. The condensed tannin has been released in intestinal mucosa to induce cytokines expression.

It is well known that there are different types of protein and polysaccharides in the cell membrane. Tannins have both hydrophobic aromatic rings and hydrophilic hydroxyl groups allowing them to bind simultaneously at several sites on the cell wall. Cell wall polysaccharides also contain hydroxyl groups as well as aromatic and glycosidic oxygen atoms that have the ability to form hydrogen bonds and hydrophobic interactions with tannin. Since, tannin-protein interactions may be specific for different tannins as well as for different proteins, to confirm which protein or polysaccharides will be attached by ACT, further studies are needed to investigate the changes of receptor production in the cell membrane. Recently, a report has identified 67-kDa laminin receptor as a cell surface receptor for the green tea constituents, (-)-epigallocatechin-3-gallate (EGCG) [118, 185].

Sirichai A. et al. have reported that grape seed, Cat's whiskers, and Sweetleaf extract significantly inhibit the activities of pancreatic α-amylase, intestinal maltase, and sucrose, respectively [186]. However, there still were no protein has been identified as a condensed tannin's receptor in the intestine.

A report indicates that extracellular dsRNA is recognized and internalized by scavenger receptor class-A (SR-A). Treatment of human epithelial cells with specific antagonists of SR-A significantly inhibited dsRNA induction of IL-6. Therefore, it is possible that ACT stimulation, in part, larger fractions procyanidins as one of the antagonists of SR-A inhibit Poly I:C induction of IL-6. However, there were no reports indicated that polyphenols interfere the stimuli of Poly I:C on SR-A as an antagonist. Furthermore, the study in Chapter 3 suggests that ACT significantly up-regulates Poly I:C-induced CXCL10 gene expression and production in MoS13 cells. Therefore, the present study denied the hypothesis that ACT acts as an antagonist of SR-A on the cell surface, interfere the binding of Poly I:C to SR-A on the cell surface, to block downstream intracellular signaling pathway.

In Chapter 2, I found that ACT and larger fractions ACT (7-mer) significantly inhibit IL-6 gene expression, production and promoter activity, whereas smaller fractions ACT (2-mer) did not effect on Poly I:C-induced IL-6 expression and production. Here, I do hypothesize that ACT and larger fractions (7-mer) bind with its special receptor to form tannin-receptor interactions on the cell membrane by hydrogen bonding and hydrophobic interactions. The interaction of ACT-receptor on cell membrane induced signaling pathway may have antagonistic or agonistic effects on Poly I:C induced MEK-, JNK-, p38-MAPKs and NF-κB signaling pathways involving in modulating cytokine expression. According to my data, I observed that ACT and ACT (7-mer) robustly inhibit the phosphorylation of IκB. The inhibitory effect ACT and ACT (7-mer) on IκB was quit much more than MEK1/2, JNK, and p38. In contrast, ACT (2-mer) increased the phosphorylation of IκB, MEK1/2 and p38. This finding suggests that the larger fraction ACT (7-mer) and the smaller fraction (2-mer) may have different effect on Poly I:C-induced signaling pathway (see Figures 2-8 and 2-9).

The biological activities of condensed tannins are recognized to be largely dependent on their molecular weight and structure. The larger fraction of condensed tannin has a large number of hydroxyl groups, has the greater binding ability compared with the smaller fraction of condensed tannin. Thus, I could conclude that apple condense tannin, especially the larger fraction ACT (7-mer) may has stronger antagonistic effect on Poly I:C-induced IκB signaling pathway via interactions of greater condensed tannin-receptor on cell membrane. In contrast,

smaller fraction ACT (2-mer) show positively regulate on Poly I:C-induced IκB and MAPKs signaling pathways as an agonist drug (Figure 5-2).

In Chapter 3, I found that ACT at a lower concentration (12.5 μg/mL) significantly up-regulated Poly I:C-induced CXCL10 production. Poly I:C bind to its receptor TLR3 resulting in activating downstream MEK-, JNK-, and p38-MAPKs signaling pathways [143]. In the present study, I investigated the effect of ACT (12.5 μg/mL) stimulation on kinase activation of MEK1/2, JNK, and p38 in Poly I:C-induced MoS13 cells. An interesting finding was that ACT (12.5 μg/mL) treatment selectively increased the phosphorylation of MEK1/2, JNK, and p38, whereas ACT (12.5 μg/mL) did not effect on IκB phosphorylation (Figure 3-5). This result indicates that lower concentration of ACT (12.5 μg/mL) did not inhibit Poly I:C-induced MAPKs signaling pathways, in contrast significantly up-regulated Poly I:C-induced PKC-MAPKs signaling pathways. These findings suggest that lower concentrations of ACT (12.5μg/mL) positively regulate Poly I:C-induced MEK1/2, JNK, and p38 signaling pathways, but do not effect on IκB signaling pathway.

Furthermore, I observed that ACT (50 μg/mL) significantly inhibit Poly I:C-induced IL-6 expression, whereas as shown in Figure 3-1 D, E, F ACT (50 μg/mL) did not inhibit Poly I:C-induced CXCL9, CXCL10, and CXCL11 expression. These results suggest that the different concentrations of ACT may induce the cytokine expression through different signaling pathways. To answer this question, I investigate the promoter sequences of IL-6 and CXCL10. On the transcriptional level, the inducible CXCL10 expression is regulated by the transcription factors IRF3, NF-κB, and AP1. Several reports suggest that dsRNA induce CXCL10 expression predominately via activation of the IRF3 signaling pathway. On the other hand, inducible IL-6 expression is mainly regulated by the transcription factors C/EBP, NF-κB, and CREB, but not IRF3. Same stimulation with ACT at different concentrations induced IL-6 and CXCL10 expression through different signaling pathway.

Therefore, I thought that IRF3 plays the key role in IL-6 and CXCL10 expression. I investigated the effect of ACT at 12.5 μg/mL and 50 μg/mL on IRF3 nuclear translocation. When compared the western blot results between the stimulation of ACT (12.5 μg/mL) and ACT (50 μg/mL), I found that ACT (12.5 μg/mL) significantly increased the nuclear translocation of IRF3. I was surprised by that ACT (50 μg/mL) did not inhibit the nuclear translocation of IRF3, whereas slightly increased IRF3 nuclear translocation. Thus, I suggest that higher concentration of ACT (50 μg/mL) down-regulates IL-6 expression predominantly via suppressing NF-κB signaling pathway, and PKC-MAPKs signaling pathways, but not via IRF3 signaling pathway.

On the other hand, lower concentration of ACT (12.5 μg/mL) up-regulate CXCL10 expression predominantly via activation of PI3K-IRF3 signaling pathways, but not via NF-κB signaling pathway. Therefore, the NF-κB and IRF3 signaling pathways are the key steps on the regulation of Poly I:C-mediated IL-6 and CXCL10 expression by ACT.

Numerous studies have shown that increased intestinal NF-κB activation in patients with inflammatory bowel disease. NF-κB, therefore, is a potential target for anti-inflammatory therapies. Accumulated studies indicate that polyphenols potent inhibit cytokine expression via suppressing NF-κB signaling pathways in intestinal epithelial cells. Here, my results indicate that ACT and larger fraction ACT (7-mer) at higher concentration inhibit IL-6 expression via predominately suppressing NF-κB signaling pathway, while ACT and smaller fraction ACT (2-mer) at lower concentration enhance MAPKs signaling pathways involving in CXCL10 expression in the mouse intestinal epithelial cells. Taken together, in Chapter 2 and Chapter 3, I have identified that ACT at different concentrations may show a different regulating effect on IL-6 and CXCL10 expression. ACT (12.5 μg/mL) up-regulated Poly I:C-induced CXCL10 gene expression predominantly via enhancing PI3K-IRF3 signaling pathways, but not via IκB signaling pathway. On the other hand, ACT (50 μg/mL) down-regulated Poly I:C induced IL-6 gene expression predominantly via suppressing IκB and MEK1/2, JNK, p38 signaling pathways.

Pomegranate is consumed fresh and in processed forms as wine and juice in Europe, Iran, Indian and other West Asian countries. The biological activity of pomegranate fruits has been widely investigated, including *in vitro, in vivo,* and clinical studies. Pomegranate peels extract has been shown to regulate the transcription of inflammatory cytokines such as IL-6, IL-8, and MCP-1 genes in the human intestinal epithelial cell line (Caco-2). However, the mechanism of that is still not very clear. In addition, there is still no study to compare the effects of pomegranate aril extract (PAE), pomegranate peel extract (PPE), and pomegranate leaf extract (PLE) on CXCL9, CXCL10, and CXCL11 expressions in intestinal epithelial cells.

In Chapter 4, I investigated the effect of pomegranate extracts on CXCL9, CXCL10, and CXCL11 expression in intestinal epithelial cells. I compared the effect of PAE, PPE and PLE on the expression of these chemokines. I observed that PPE significantly increased CXCL10 gene expression, production, and promoter activity via activation of both PKC, MEK1/2-ERK1/2, JNK signaling pathways and PI3K-IRF3 signaling pathway. However, PAE and PLE did not affect on CXCL10 expression.

In addition, I confirmed the enhancement effect of PPE CXCL10 expression *in vivo*. However, PAE and PLE did not induce CXCL10 gene expression. These findings suggest that PAE, PPE, and PLE may have different effects on cytokine expression in intestinal epithelial cells. In this study, all of this 80% acetone extracts from pomegranate arils, peels and leaves are the mixtures of polyphenol components, they are not one or two pure compounds. I have characterized the phenolic components of PAE, PPE, and PLE by HPLC analysis. The results showed that PAE contains some phenolic components characterized, the main components are ellagitannin monomer Punicalagin, Punicalin and ellagitannin oligomer oenothein B and eucalbanin B (see Figures 4-1 and 4-2). Punicalagin is the main phenolics in pomegranate PPE. PLE contains only one main hydrolysable tannin, strictinin A. These results suggest that the differences of phenolic components of different pomegranate fruit parts result in the different effect on CXCL9, CXCL10, and CXCL11 expression of intestinal epithelial cells.

The biological activities of PPE and punicalagin have been widely investigated. It has been reported that pomegranate peel can help prevent or treat various disease risk factors including oxidative stress and inflammatory activities. It has been reported that PPE may inhibit numbers of inflammatory cytokines expression such as IL-6 and IL-8. In addition, punicalagin has been shown to have anti-proliferative, anti-inflammatory properties *in vivo* and *in vitro*. Furthermore, accumulated studies suggest that the pharmacological activities from pomegranate peels have been shown to be associated with the main phenolic component, punicalatin.

Here, I am the first to show that PPE significantly up-regulate CXCL10 expression of intestinal epithelial cells *in vivo* and *in vitro*. It has been reported that PPE has the highest concentration of punicalagin. I previously quantified the content of punicalagin in PPE. The punicalagin contents in the PPE and PAE have been quantified to be approximately 70 mg/100g. However, in the present study, it is still unknown whether the main phenolic component punucalagin in PPE may up-regulate CXCL10 expression of intestinal epithelial cells. Further studies are needed to examine the enhancement effect of punicalagin on CXCL10 expression of intestinal epithelial cells *in vivo* and *in vitro*.

Punicalagin is a hydrolysable tannin and isomers of 2,3-(*S*)-hexahydroxydiphenoyl-4,6-(*S*,*S*)-gallagyl-D-glucose (see Figure 4-2). Since punicalagin are water soluble, it will be hydrolyzed to smaller polyphenolic compounds in the small intestine under normal physiological conditions. It has been reported that punicalagin inhibit inflammatory cytokine NO, PGE2, IL-1β, IL-6 and TNF-α expression via suppression of toll-like receptor 4-mediated MAPKs and NF-ϰB activation in macrophages. [187]. Xu. XiaoL. et al., have also reported that punicalagin induces Nrf2/HO-1 expression via up-regulation of

PI3K/AKT pathway [188]. Another report also indicated that punicalagin inhibit IPS-induced TNF-α and IL-6 expression via inhibition of phosphorylation of IκB, p38, JNK and nuclear translocation of p65 subunit in the microglia [189]. Punicalagin has also been shown to down-regulate LPS-induced production of IL-6, IL-8, and TNF-α via inhibition of NF-κB signaling pathway and MAPKs (p38, JNK, ERK) signaling pathway in bovine endometrial epithelial cells (bEECs). In the present study, I found that PPE significantly increased CXCL10 gene expression and production. I also observed that PPE treatment up-regulated the phosphorylation of PKC, MEK1/2, ERK1/2, and JNK, and the nuclear translocation of IRF3 in intestinal epithelial cells. Therefore, I hypothesis that punicalagin may increase CXCL10 expression via activation of phosphorylation of MEK1/2, ERK1/2, and JNK, and nuclear translocation of IRF3.

In addition, a recent report suggests that CXCL10 expression may be regulated by PKC signaling pathway. Couples of previous studies have suggested that phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays a central role in modulating diverse downstream signaling pathways. It has been reported that influenza virus infection modulated CXCL10 expression via activation of the PI3K/Akt pathway. Furthermore, several reports suggest that PI3K specific inhibitor LY294002 and PKC specific inhibitor (RO31-8220) could suppress the CXCL10 expression. This evidence indicates that the PKC and PI3K/Akt pathway plays an important role in CXCL10 expression. In the present study, I observed that inhibitors of PI3K and PKC significantly suppressed PPE-induced CXCL10 expression. These findings suggest that PPE up-regulates CXCL10 gene expression via activation of PKC and PI3K signaling pathway signaling pathway. Taken together, PPE may up-regulate CXCL10 expression via activation of PKC-MEK1/2, ERK1/2, JNK signaling pathway, and PI3K-IRF3 signaling pathway in intestinal epithelial cells.

It has been reported that tannins could bind to a protein via hydrogen binding to form tannin-protein interactions. Hydrogen bonding between phenolic hydroxyl and peptide carbonyl is major force stabilizing condensed tannin-protein complexes. A recent report has identified a laminin receptor as a cell surface receptor for the green tea constituents, (-)-epigallocatechin-3-gallate (EGCG). However, it is still not clear about the punicalagin receptor. Here, I hypothesis that punicalagin act on a special receptor on the cell surface to induce PKC, MEK1/2, ERK1/2, JNK signaling pathway and PI3K-IRF3 signaling pathway, involving in CXCL10 expression of intestinal epithelia cells (see Figure 5-3). In summary, in this chapter, my results first indicate that PPE may increase CXCL10 expression *in vivo* and *in vitro* in mouse intestinal epithelial cells, predominately via activation of PI3K-IRF3 signaling pathway and PKC-MAPKs (MEK1/2, ERK1/2, JNK) signaling pathway, but now NF-κB

signaling pathway.

In conclusion

In this whole works, I have investigated the effect of ACT on IL-6 and CXCL10 gene expression, and also compared the effect of PAE, PPE, and PLE on CXCL10 gene expression in mouse intestinal epithelial cells. It was observed that ACT showed different regulating effect on IL-6 and CXCL10 gene expression in Poly I:C-induced MoS13 cells. Here, I suggest that higher concentration of ACT (50 μg/mL) inhibit IL-6 expression predominantly via suppressing IκB signaling pathway, and PKC-MAPKs (MEK1/2, JNK, p38) signaling pathways. In contrast, in Chapter 2, I first found that lower concentration of ACT (12.5 μg/mL) up-regulated Poly I:C-induced CXCL10 expression predominantly via activation of PI3K-IRF3 signaling pathways, but not via IκB signaling pathway. In Chapter 3, PPE was found to up-regulate CXCL10 expression predominantly via activation of PI3K-IRF3 signaling pathway and PKC-MAPKs (MEK1/2, ERK1/2, and JNK) signaling pathway. Whereas PPE did not induce IκB signaling pathway. On the transcriptional level, there is no binding site for IRF3 in the IL-6 promoter region, while IRF3 play a central role in CXCL10 gene transcription. These results indicate that ACT and PPE may predominantly up-regulate PI3K-IRF3 signaling pathway, involving in increasing of CXCL10 expression, while ACT may predominantly down-regulate IκB signaling pathway, resulting in the decreasing of IL-6 expression.

These findings suggest that apple peel extract and pomegranate peel extract may regulate cytokine and chemokine expression in intestinal epithelial cells via different signaling pathways. These results also support that consumption of apples and pomegranate may help protect against intestine disorder by decreasing pro-inflammatory cytokines and enhancing gut immunity with increasing chemokines in the normal steady state.

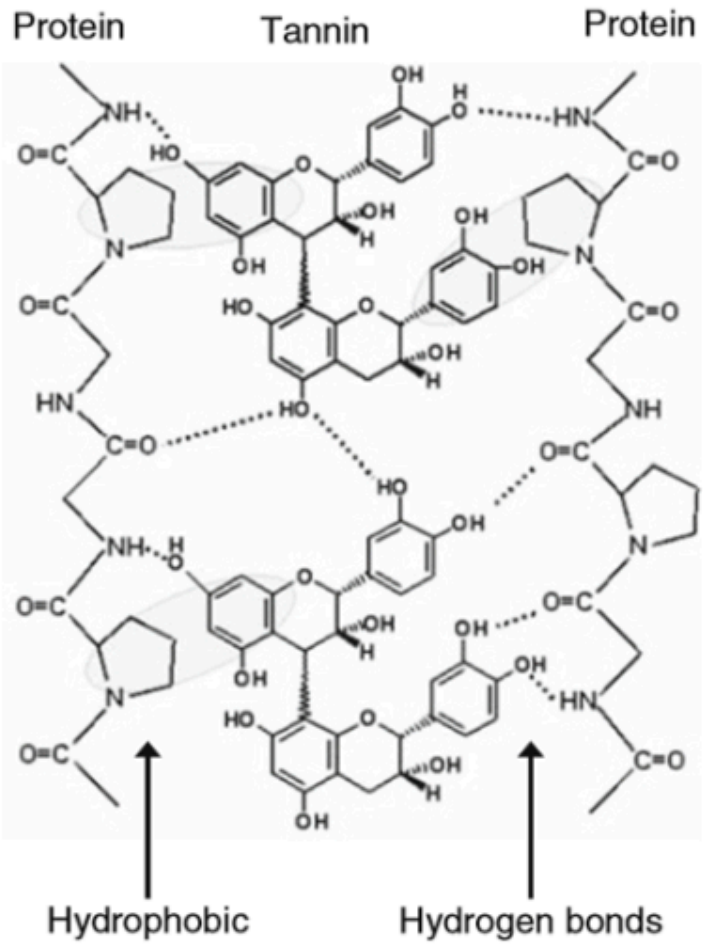


Figure 5-1 The interaction between tannins and protein (*Santos-Buelga and Freitas 2009*)

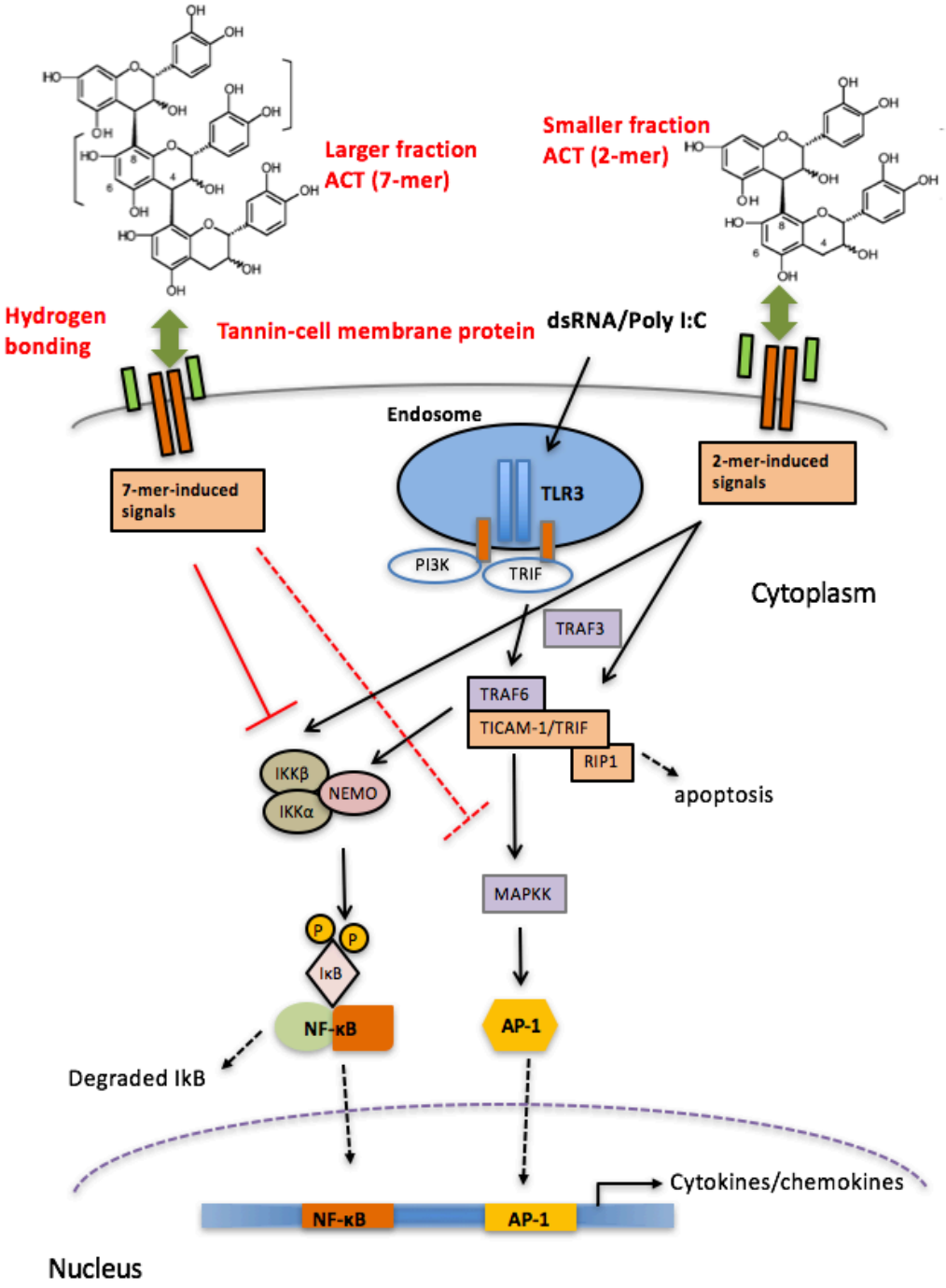


Figure 5-2 Effect of larger fraction ACT (7-mer) and smaller fraction ACT (2-mer) on Poly I:C-induced signal transduction pathways

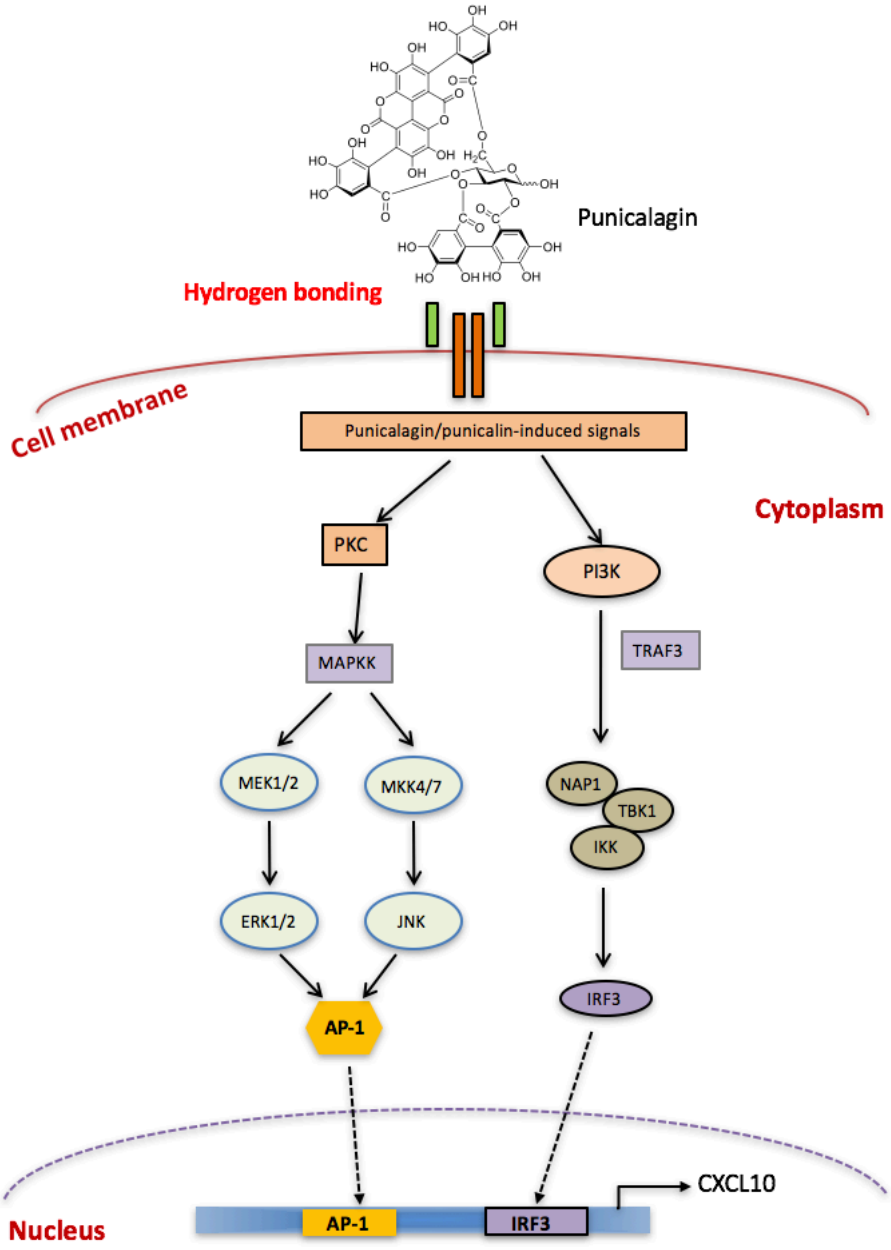


Figure 5-3 Effect of Punucalagin on Poly I:C-induced signal transduction pathways

7 References

1. Baumgart, D. C. & Sandborn, W. J. (2007) Inflammatory bowel disease: clinical aspects and established and evolving therapies, *Lancet*. **369**, 1641-57.
2. Duricova, D., Burisch, J., Jess, T., Gower-Rousseau, C., Lakatos, P. L. & ECCO-EpiCom (2014) Age-related differences in presentation and course of inflammatory bowel disease: an update on the population-based literature, *J Crohns Colitis*. **8**, 1351-61.
3. Annese, V., Latiano, A. & Andriulli, A. (2003) Genetics of inflammatory bowel disease: the beginning of the end or the end of the beginning?, *Dig Liver Dis*. **35**, 442-9.
4. Bouma, G. & Strober, W. (2003) The immunological and genetic basis of inflammatory bowel disease, *Nat Rev Immunol*. **3**, 521-33.
5. Keshavarzian, A., Fusunyan, R. D., Jacyno, M., Winship, D., MacDermott, R. P. & Sanderson, I. R. (1999) Increased interleukin-8 (IL-8) in rectal dialysate from patients with ulcerative colitis: evidence for a biological role for IL-8 in inflammation of the colon, *Am J Gastroenterol*. **94**, 704-12.
6. Hébuterne, X., Filippi, J., Al-Jaouni, R. & Schneider, S. (2009) Nutritional consequences and nutrition therapy in Crohn's disease, *Gastroenterol Clin Biol*. **33 Suppl 3**, S235-44.
7. Png, C. W., Lindén, S. K., Gilshenan, K. S., Zoetendal, E. G., McSweeney, C. S., Sly, L. I., McGuckin, M. A. & Florin, T. H. (2010) Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria, *Am J Gastroenterol*. **105**, 2420-8.
8. Kangro, H. O., Chong, S. K., Hardiman, A., Heath, R. B. & Walker-Smith, J. A. (1990) A prospective study of viral and mycoplasma infections in chronic inflammatory bowel disease, *Gastroenterology*. **98**, 549-53.
9. Khan, R. R., Lawson, A. D., Minnich, L. L., Martin, K., Nasir, A., Emmett, M. K., Welch, C. A. & Udall, J. N. (2009) Gastrointestinal norovirus infection associated with exacerbation of inflammatory bowel disease, *J Pediatr Gastroenterol Nutr.* **48**, 328-33.
10. Westall, F. C. (2006) Integrating theories of the etiology of Crohn's disease on the etiology of Crohn's disease: questioning the hypotheses. William M. Chamberlin, Saleh A. Naser Med Sci Monit, 2006; 12(2): RA27-33, *Med Sci Monit*. **12**, LE5-6.
11. Stucchi, A., Reed, K., O'Brien, M., Cerda, S., Andrews, C., Gower, A., Bushell, K., Amar, S., Leeman, S. & Becker, J. (2006) A new transcription factor that regulates TNF-alpha gene expression, LITAF, is increased in intestinal tissues from patients with CD and UC, *Inflamm Bowel Dis*. **12**, 581-7.
12. Yamamoto-Furusho, J. K. (2007) Innovative therapeutics for inflammatory bowel disease, *World J Gastroenterol*. **13**, 1893-6.
13. Podolsky, D. K. (2002) Inflammatory bowel disease, *N Engl J Med*. **347**, 417-29.

14. Shanahan, F. (2002) Crohn's disease, *Lancet*. **359**, 62-9.
15. MacDonald, T. T. (2003) The mucosal immune system, *Parasite Immunol*. **25**, 235-46.
16. Mitsuyama, K., Toyonaga, A., Sasaki, E., Ishida, O., Ikeda, H., Tsuruta, O., Harada, K., Tateishi, H., Nishiyama, T. & Tanikawa, K. (1995) Soluble interleukin-6 receptors in inflammatory bowel disease: relation to circulating interleukin-6, *Gut*. **36**, 45-9.
17. Reinisch, W., Gasché, C., Tillinger, W., Wyatt, J., Lichtenberger, C., Willheim, M., Dejaco, C., Waldhör, T., Bakos, S., Vogelsang, H., Gangl, A. & Lochs, H. (1999) Clinical relevance of serum interleukin-6 in Crohn's disease: single point measurements, therapy monitoring, and prediction of clinical relapse, *Am J Gastroenterol*. **94**, 2156-64.
18. Yamamoto, M., Yoshizaki, K., Kishimoto, T. & Ito, H. (2000) IL-6 is required for the development of Th1 cell-mediated murine colitis, *J Immunol*. **164**, 4878-82.
19. Kallen, K. J. (2002) The role of transsignalling via the agonistic soluble IL-6 receptor in human diseases, *Biochim Biophys Acta*. **1592**, 323-43.
20. Bauer, J., Bauer, T. M., Kalb, T., Taga, T., Lengyel, G., Hirano, T., Kishimoto, T., Acs, G., Mayer, L. & Gerok, W. (1989) Regulation of interleukin 6 receptor expression in human monocytes and monocyte-derived macrophages. Comparison with the expression in human hepatocytes, *J Exp Med*. **170**, 1537-49.
21. Freeman, G. J., Freedman, A. S., Rabinowe, S. N., Segil, J. M., Horowitz, J., Rosen, K., Whitman, J. F. & Nadler, L. M. (1989) Interleukin 6 gene expression in normal and neoplastic B cells, *J Clin Invest*. **83**, 1512-8.
22. Jirik, F. R., Podor, T. J., Hirano, T., Kishimoto, T., Loskutoff, D. J., Carson, D. A. & Lotz, M. (1989) Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells, *J Immunol*. **142**, 144-7.
23. Sironi, M., Breviario, F., Proserpio, P., Biondi, A., Vecchi, A., Van Damme, J., Dejana, E. & Mantovani, A. (1989) IL-1 stimulates IL-6 production in endothelial cells, *J Immunol*. **142**, 549-53.
24. Brinson, C. W., Lu, Z., Li, Y., Lopes-Virella, M. F. & Huang, Y. (2016) Lipopolysaccharide and IL-1 β coordinate a synergy on cytokine production by upregulating MyD88 expression in human gingival fibroblasts, *Mol Immunol*. **79**, 47-54.
25. Bédard, M., McClure, C. D., Schiller, N. L., Francoeur, C., Cantin, A. & Denis, M. (1993) Release of interleukin-8, interleukin-6, and colony-stimulating factors by upper airway epithelial cells: implications for cystic fibrosis, *Am J Respir Cell Mol Biol*. **9**, 455-62.
26. Asokananthan, N., Graham, P. T., Fink, J., Knight, D. A., Bakker, A. J., McWilliam, A. S., Thompson, P. J. & Stewart, G. A. (2002) Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells, *J Immunol*. **168**, 3577-85.

27. McGee, D. W., Beagley, K. W., Aicher, W. K. & McGhee, J. R. (1993) Transforming growth factor-beta and IL-1 beta act in synergy to enhance IL-6 secretion by the intestinal epithelial cell line, IEC-6, *J Immunol*. **151**, 970-8.
28. Parikh, A. A., Salzman, A. L., Fischer, J. E., Szabó, C. & Hasselgren, P. O. (1997) Interleukin-1 beta and interferon-gamma regulate interleukin-6 production in cultured human intestinal epithelial cells, *Shock*. **8**, 249-55.
29. Into, T., Inomata, M., Shibata, K. & Murakami, Y. (2010) Effect of the antimicrobial peptide LL-37 on Toll-like receptors 2-, 3- and 4-triggered expression of IL-6, IL-8 and CXCL10 in human gingival fibroblasts, *Cell Immunol*. **264**, 104-9.
30. Riedemann, N. C., Guo, R. F., Hollmann, T. J., Gao, H., Neff, T. A., Reuben, J. S., Speyer, C. L., Sarma, J. V., Wetsel, R. A., Zetoune, F. S. & Ward, P. A. (2004) Regulatory role of C5a in LPS-induced IL-6 production by neutrophils during sepsis, *FASEB J*. **18**, 370-2.
31. Akira, S. (1997) IL-6-regulated transcription factors, *Int J Biochem Cell Biol*. **29**, 1401-18.
32. Son, Y. H., Jeong, Y. T., Lee, K. A., Choi, K. H., Kim, S. M., Rhim, B. Y. & Kim, K. (2008) Roles of MAPK and NF-kappaB in interleukin-6 induction by lipopolysaccharide in vascular smooth muscle cells, *J Cardiovasc Pharmacol*. **51**, 71-7.
33. Luo, G., Hershko, D. D., Robb, B. W., Wray, C. J. & Hasselgren, P. O. (2003) IL-1beta stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF-kappa B, *Am J Physiol Regul Integr Comp Physiol*. **284**, R1249-54.
34. Tsutsumi, T., Suzuki, T., Moriya, K., Shintani, Y., Fujie, H., Miyoshi, H., Matsuura, Y., Koike, K. & Miyamura, T. (2003) Hepatitis C virus core protein activates ERK and p38 MAPK in cooperation with ethanol in transgenic mice, *Hepatology*. **38**, 820-8.
35. Castro, V., Murillo, R., Klaas, C. A., Meunier, C., Mora, G., Pahl, H. L. & Merfort, I. (2000) Inhibition of the transcription factor NF-kappa B by sesquiterpene lactones from Podachaenium eminens, *Planta Med*. **66**, 591-5.
36. Afaq, F., Adhami, V. M., Ahmad, N. & Mukhtar, H. (2003) Inhibition of ultraviolet B-mediated activation of nuclear factor kappaB in normal human epidermal keratinocytes by green tea Constituent (-)-epigallocatechin-3-gallate, *Oncogene*. **22**, 1035-44.
37. Yang, F., Oz, H. S., Barve, S., de Villiers, W. J., McClain, C. J. & Varilek, G. W. (2001) The green tea polyphenol (-)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal epithelial cell line IEC-6, *Mol Pharmacol*. **60**, 528-33.
38. Laing, K. J. & Secombes, C. J. (2004) Chemokines, *Dev Comp Immunol*. **28**, 443-60.
39. Gerszten, R. E., Garcia-Zepeda, E. A., Lim, Y. C., Yoshida, M., Ding, H. A., Gimbrone, M. A., Luster, A. D., Luscinskas, F. W. & Rosenzweig, A. (1999) MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions, *Nature*. **398**,

718-23.

40. Lukacs, N. W. (2001) Role of chemokines in the pathogenesis of asthma, *Nat Rev Immunol*. **1**, 108-16.
41. MacDermott, R. P., Sanderson, I. R. & Reinecker, H. C. (1998) The central role of chemokines (chemotactic cytokines) in the immunopathogenesis of ulcerative colitis and Crohn's disease, *Inflamm Bowel Dis*. **4**, 54-67.
42. Papadakis, K. A. & Targan, S. R. (2000) The role of chemokines and chemokine receptors in mucosal inflammation, *Inflamm Bowel Dis*. **6**, 303-13.
43. Uguccioni, M., Gionchetti, P., Robbiani, D. F., Rizzello, F., Peruzzo, S., Campieri, M. & Baggiolini, M. (1999) Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis, *Am J Pathol*. **155**, 331-6.
44. Loetscher, M., Gerber, B., Loetscher, P., Jones, S. A., Piali, L., Clark-Lewis, I., Baggiolini, M. & Moser, B. (1996) Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes, *J Exp Med*. **184**, 963-9.
45. Qin, S., Rottman, J. B., Myers, P., Kassam, N., Weinblatt, M., Loetscher, M., Koch, A. E., Moser, B. & Mackay, C. R. (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions, *J Clin Invest*. **101**, 746-54.
46. Chew, V., Chen, J., Lee, D., Loh, E., Lee, J., Lim, K. H., Weber, A., Slankamenac, K., Poon, R. T., Yang, H., Ooi, L. L., Toh, H. C., Heikenwalder, M., Ng, I. O., Nardin, A. & Abastado, J. P. (2012) Chemokine-driven lymphocyte infiltration: an early intratumoural event determining long-term survival in resectable hepatocellular carcinoma, *Gut*. **61**, 427-38.
47. Sauty, A., Dziejman, M., Taha, R. A., Iarossi, A. S., Neote, K., Garcia-Zepeda, E. A., Hamid, Q. & Luster, A. D. (1999) The T cell-specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells, *J Immunol*. **162**, 3549-58.
48. Clarke, D. L., Clifford, R. L., Jindarat, S., Proud, D., Pang, L., Belvisi, M. & Knox, A. J. (2010) TNF α and IFN γ synergistically enhance transcriptional activation of CXCL10 in human airway smooth muscle cells via STAT-1, NF- κ B, and the transcriptional coactivator CREB-binding protein, *J Biol Chem*. **285**, 29101-10.
49. Tudhope, S. J., Catley, M. C., Fenwick, P. S., Russell, R. E., Rumsey, W. L., Newton, R., Barnes, P. J. & Donnelly, L. E. (2007) The role of IkappaB kinase 2, but not activation of NF-kappaB, in the release of CXCR3 ligands from IFN-gamma-stimulated human bronchial epithelial cells, *J Immunol*. **179**, 6237-45.
50. Harvey, C. E., Post, J. J., Palladinetti, P., Freeman, A. J., Ffrench, R. A., Kumar, R. K., Marinos, G. & Lloyd, A. R. (2003) Expression of the chemokine IP-10 (CXCL10) by hepatocytes in chronic hepatitis C virus infection correlates with histological severity and

lobular inflammation, *J Leukoc Biol*. **74**, 360-9.

51. Spurrell, J. C., Wiehler, S., Zaheer, R. S., Sanders, S. P. & Proud, D. (2005) Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection, *Am J Physiol Lung Cell Mol Physiol*. **289**, L85-95.
52. Ostvik, A. E., Granlund, A. V., Bugge, M., Nilsen, N. J., Torp, S. H., Waldum, H. L., Damås, J. K., Espevik, T. & Sandvik, A. K. (2013) Enhanced expression of CXCL10 in inflammatory bowel disease: potential role of mucosal Toll-like receptor 3 stimulation, *Inflamm Bowel Dis*. **19**, 265-74.
53. Cliffe, L. J., Humphreys, N. E., Lane, T. E., Potten, C. S., Booth, C. & Grencis, R. K. (2005) Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion, *Science*. **308**, 1463-5.
54. Dwinell, M. B., Lügering, N., Eckmann, L. & Kagnoff, M. F. (2001) Regulated production of interferon-inducible T-cell chemoattractants by human intestinal epithelial cells, *Gastroenterology*. **120**, 49-59.
55. Shibahara, T., Wilcox, J. N., Couse, T. & Madara, J. L. (2001) Characterization of epithelial chemoattractants for human intestinal intraepithelial lymphocytes, *Gastroenterology*. **120**, 60-70.
56. Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3, *Nature*. **413**, 732-8.
57. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K. & Akira, S. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway, *Science*. **301**, 640-3.
58. Kawai, T., Adachi, O., Ogawa, T., Takeda, K. & Akira, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin, *Immunity*. **11**, 115-22.
59. Meylan, E., Burns, K., Hofmann, K., Blancheteau, V., Martinon, F., Kelliher, M. & Tschopp, J. (2004) RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation, *Nat Immunol*. **5**, 503-7.
60. Han, K. J., Su, X., Xu, L. G., Bin, L. H., Zhang, J. & Shu, H. B. (2004) Mechanisms of the TRIF-induced interferon-stimulated response element and NF-kappaB activation and apoptosis pathways, *J Biol Chem*. **279**, 15652-61.
61. Gohda, J., Matsumura, T. & Inoue, J. (2004) Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not toll/IL-1 receptor domain-containing adaptor-inducing IFN-beta (TRIF)-dependent pathway in TLR signaling, *J Immunol*. **173**, 2913-7.
62. Ramnath, R. D., Sun, J., Adhikari, S., Zhi, L. & Bhatia, M. (2008) Role of PKC-delta on substance P-induced chemokine synthesis in pancreatic acinar cells, *Am J Physiol Cell*

Physiol. **294**, C683-92.

63. Johnsen, S. P., Overvad, K., Stripp, C., Tjønneland, A., Husted, S. E. & Sørensen, H. T. (2003) Intake of fruit and vegetables and the risk of ischemic stroke in a cohort of Danish men and women, *Am J Clin Nutr.* **78**, 57-64.
64. Rissanen, T. H., Voutilainen, S., Virtanen, J. K., Venho, B., Vanharanta, M., Mursu, J. & Salonen, J. T. (2003) Low intake of fruits, berries and vegetables is associated with excess mortality in men: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study, *J Nutr.* **133**, 199-204.
65. Temple, N. J. & Gladwin, K. K. (2003) Fruit, vegetables, and the prevention of cancer: research challenges, *Nutrition*. **19**, 467-70.
66. Akiyama, H., Sakushima, J., Taniuchi, S., Kanda, T., Yanagida, A., Kojima, T., Teshima, R., Kobayashi, Y., Goda, Y. & Toyoda, M. (2000) Antiallergic effect of apple polyphenols on the allergic model mouse, *Biol Pharm Bull*. **23**, 1370-3.
67. Kanda, T., Akiyama, H., Yanagida, A., Tanabe, M., Goda, Y., Toyoda, M., Teshima, R. & Saito, Y. (1998) Inhibitory effects of apple polyphenol on induced histamine release from RBL-2H3 cells and rat mast cells, *Biosci Biotechnol Biochem*. **62**, 1284-9.
68. Ohnishi-Kameyama, M., Yanagida, A., Kanda, T. & Nagata, T. (1997) Identification of catechin oligomers from apple (Malus pumila cv. Fuji) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry, *Rapid Commun Mass Spectrom*. **11**, 31-6.
69. Sun, J., Chu, Y. F., Wu, X. & Liu, R. H. (2002) Antioxidant and antiproliferative activities of common fruits, *J Agric Food Chem*. **50**, 7449-54.
70. Fathy, S. M. & Drees, E. A. (2016) Protective effects of Egyptian cloudy apple juice and apple peel extract on lipid peroxidation, antioxidant enzymes and inflammatory status in diabetic rat pancreas, *BMC Complement Altern Med*. **16**, 8.
71. Figueroa-Espinoza, M. C., Zafimahova, A., Alvarado, P. G., Dubreucq, E. & Poncet-Legrand, C. (2015) Grape seed and apple tannins: emulsifying and antioxidant properties, *Food Chem*. **178**, 38-44.
72. Vineetha, V. P., Girija, S., Soumya, R. S. & Raghu, K. G. (2014) Polyphenol-rich apple (Malus domestica L.) peel extract attenuates arsenic trioxide induced cardiotoxicity in H9c2 cells via its antioxidant activity, *Food Funct*. **5**, 502-11.
73. Denis, M. C., Furtos, A., Dudonné, S., Montoudis, A., Garofalo, C., Desjardins, Y., Delvin, E. & Levy, E. (2013) Apple peel polyphenols and their beneficial actions on oxidative stress and inflammation, *PLoS One*. **8**, e53725.
74. Wolfe, K., Wu, X. & Liu, R. H. (2003) Antioxidant activity of apple peels, *J Agric Food Chem*. **51**, 609-14.
75. McCann, M. J., Gill, C. I., O' Brien, G., Rao, J. R., McRoberts, W. C., Hughes, P., McEntee,

R. & Rowland, I. R. (2007) Anti-cancer properties of phenolics from apple waste on colon carcinogenesis in vitro, *Food Chem Toxicol*. **45**, 1224-30.

76. Yoon, H. & Liu, R. H. (2007) Effect of selected phytochemicals and apple extracts on NF-kappaB activation in human breast cancer MCF-7 cells, *J Agric Food Chem*. **55**, 3167-73.
77. Carrasco-Pozo, C., Speisky, H., Brunser, O., Pastene, E. & Gotteland, M. (2011) Apple peel polyphenols protect against gastrointestinal mucosa alterations induced by indomethacin in rats, *J Agric Food Chem*. **59**, 6459-66.
78. Ercisli, S., Gadze, J., Agar, G., Yildirim, N. & Hizarci, Y. (2011) Genetic relationships among wild pomegranate (Punica granatum) genotypes from Coruh Valley in Turkey, *Genet Mol Res*. **10**, 459-64.
79. Krueger, D. A. (2012) Composition of pomegranate juice, *J AOAC Int*. **95**, 163-8.
80. Adams, L. S., Seeram, N. P., Aggarwal, B. B., Takada, Y., Sand, D. & Heber, D. (2006) Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells, *J Agric Food Chem*. **54**, 980-5.
81. Aviram, M., Volkova, N., Coleman, R., Dreher, M., Reddy, M. K., Ferreira, D. & Rosenblat, M. (2008) Pomegranate phenolics from the peels, arils, and flowers are antiatherogenic: studies in vivo in atherosclerotic apolipoprotein e-deficient (E 0) mice and in vitro in cultured macrophages and lipoproteins, *J Agric Food Chem*. **56**, 1148-57.
82. Mohan, M., Waghulde, H. & Kasture, S. (2010) Effect of pomegranate juice on Angiotensin II-induced hypertension in diabetic Wistar rats, *Phytother Res*. **24 Suppl 2**, S196-203.
83. Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., Holcroft, D. M. & Kader, A. A. (2000) Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing, *J Agric Food Chem*. **48**, 4581-9.
84. Azadzoi, K. M., Schulman, R. N., Aviram, M. & Siroky, M. B. (2005) Oxidative stress in arteriogenic erectile dysfunction: prophylactic role of antioxidants, *J Urol*. **174**, 386-93.
85. Rosenblat, M., Volkova, N., Attias, J., Mahamid, R. & Aviram, M. (2010) Consumption of polyphenolic-rich beverages (mostly pomegranate and black currant juices) by healthy subjects for a short term increased serum antioxidant status, and the serum's ability to attenuate macrophage cholesterol accumulation, *Food Funct*. **1**, 99-109.
86. Ito, H., Li, P., Koreishi, M., Nagatomo, A., Nishida, N. & Yoshida, T. (2014) Ellagitannin oligomers and a neolignan from pomegranate arils and their inhibitory effects on the formation of advanced glycation end products, *Food Chem*. **152**, 323-30.
87. Hollebeeck, S., Winand, J., Hérent, M. F., During, A., Leclercq, J., Larondelle, Y. & Schneider, Y. J. (2012) Anti-inflammatory effects of pomegranate (Punica granatum L.) husk ellagitannins in Caco-2 cells, an in vitro model of human intestine, *Food Funct*. **3**, 875-85.

88. Lei, F., Xing, D. M., Xiang, L., Zhao, Y. N., Wang, W., Zhang, L. J. & Du, L. J. (2003) Pharmacokinetic study of ellagic acid in rat after oral administration of pomegranate leaf extract, *J Chromatogr B Analyt Technol Biomed Life Sci*. **796**, 189-94.

89. Lei, F., Zhang, X. N., Wang, W., Xing, D. M., Xie, W. D., Su, H. & Du, L. J. (2007) Evidence of anti-obesity effects of the pomegranate leaf extract in high-fat diet induced obese mice, *Int J Obes (Lond)*. **31**, 1023-9.

90. Adhami, V. M. & Mukhtar, H. (2006) Polyphenols from green tea and pomegranate for prevention of prostate cancer, *Free Radic Res*. **40**, 1095-104.

91. Bekir, J., Mars, M., Souchard, J. P. & Bouajila, J. (2013) Assessment of antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of pomegranate (Punica granatum) leaves, *Food Chem Toxicol*. **55**, 470-5.

92. Block, G., Patterson, B. & Subar, A. (1992) Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence, *Nutr Cancer*. **18**, 1-29.

93. Amre, D. K., D'Souza, S., Morgan, K., Seidman, G., Lambrette, P., Grimard, G., Israel, D., Mack, D., Ghadirian, P., Deslandres, C., Chotard, V., Budai, B., Law, L., Levy, E. & Seidman, E. G. (2007) Imbalances in dietary consumption of fatty acids, vegetables, and fruits are associated with risk for Crohn's disease in children, *Am J Gastroenterol*. **102**, 2016-25.

94. Knekt, P., Jarvinen, R., Reunanen, A. & Maatela, J. (1996) Flavonoid intake and coronary mortality in Finland: a cohort study, *BMJ*. **312**, 478-81.

95. Knekt, P., Järvinen, R., Seppänen, R., Hellövaara, M., Teppo, L., Pukkala, E. & Aromaa, A. (1997) Dietary flavonoids and the risk of lung cancer and other malignant neoplasms, *Am J Epidemiol*. **146**, 223-30.

96. Tabak, C., Arts, I. C., Smit, H. A., Heederik, D. & Kromhout, D. (2001) Chronic obstructive pulmonary disease and intake of catechins, flavonols, and flavones: the MORGEN Study, *Am J Respir Crit Care Med*. **164**, 61-4.

97. Eberhardt, M. V., Lee, C. Y. & Liu, R. H. (2000) Antioxidant activity of fresh apples, *Nature*. **405**, 903-4.

98. Boyer, J. & Liu, R. H. (2004) Apple phytochemicals and their health benefits, *Nutr J*. **3**, 5.

99. Gossé, F., Roussi, S., Guyot, S., Schoenfelder, A., Mann, A., Bergerat, J. P., Seiler, N. & Raul, F. (2006) Potentiation of apple procyanidin-triggered apoptosis by the polyamine oxidase inactivator MDL 72527 in human colon cancer-derived metastatic cells, *Int J Oncol*. **29**, 423-8.

100. Miura, T., Chiba, M., Kasai, K., Nozaka, H., Nakamura, T., Shoji, T., Kanda, T., Ohtake, Y. & Sato, T. (2008) Apple procyanidins induce tumor cell apoptosis through mitochondrial pathway activation of caspase-3, *Carcinogenesis*. **29**, 585-93.

101. Seiler, N., Chaabi, M., Roussi, S., Gossé, F., Lobstein, A. & Raul, F. (2006) Synergism

between apple procyanidins and lysosomotropic drugs: potential in chemoprevention, *Anticancer Res*. **26**, 3381-5.

102. Skyberg, J. A., Robison, A., Golden, S., Rollins, M. F., Callis, G., Huarte, E., Kochetkova, I., Jutila, M. A. & Pascual, D. W. (2011) Apple polyphenols require T cells to ameliorate dextran sulfate sodium-induced colitis and dampen proinflammatory cytokine expression, *J Leukoc Biol*. **90**, 1043-54.

103. Terra, X., Palozza, P., Fernandez-Larrea, J., Ardevol, A., Blade, C., Pujadas, G., Salvado, J., Arola, L. & Blay, M. T. (2011) Procyanidin dimer B1 and trimer C1 impair inflammatory response signalling in human monocytes, *Free Radic Res*. **45**, 611-9.

104. Zampetaki, A., Zhang, Z., Hu, Y. & Xu, Q. (2005) Biomechanical stress induces IL-6 expression in smooth muscle cells via Ras/Rac1-p38 MAPK-NF-kappaB signaling pathways, *Am J Physiol Heart Circ Physiol*. **288**, H2946-54.

105. Libermann, T. A. & Baltimore, D. (1990) Activation of interleukin-6 gene expression through the NF-kappa B transcription factor, *Mol Cell Biol*. **10**, 2327-34.

106. Schaefer, T. M., Fahey, J. V., Wright, J. A. & Wira, C. R. (2005) Innate immunity in the human female reproductive tract: antiviral response of uterine epithelial cells to the TLR3 agonist poly(I:C), *J Immunol*. **174**, 992-1002.

107. Hatano, R., Yamada, K., Iwamoto, T., Maeda, N., Emoto, T., Shimizu, M. & Totsuka, M. (2013) Antigen presentation by small intestinal epithelial cells uniquely enhances IFN-γ secretion from CD4+ intestinal intraepithelial lymphocytes, *Biochem Biophys Res Commun*. **435**, 592-6.

108. Tadaki, H., Arakawa, H., Mizuno, T., Suzuki, T., Takeyama, K., Mochizuki, H., Tokuyama, K., Yokota, S. & Morikawa, A. (2009) Double-stranded RNA and TGF-alpha promote MUC5AC induction in respiratory cells, *J Immunol*. **182**, 293-300.

109. Lee, Y., Kim, H., Kim, S., Kim, K. H. & Chung, J. H. (2010) Activation of toll-like receptors 2, 3 or 5 induces matrix metalloproteinase-1 and -9 expression with the involvement of MAPKs and NF-kappaB in human epidermal keratinocytes, *Exp Dermatol*. **19**, e44-9.

110. Lebre, M. C., van der Aar, A. M., van Baarsen, L., van Capel, T. M., Schuitemaker, J. H., Kapsenberg, M. L. & de Jong, E. C. (2007) Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9, *J Invest Dermatol*. **127**, 331-41.

111. Davey, M. P., Martin, T. M., Planck, S. R., Lee, J., Zamora, D. & Rosenbaum, J. T. (2006) Human endothelial cells express NOD2/CARD15 and increase IL-6 secretion in response to muramyl dipeptide, *Microvasc Res*. **71**, 103-7.

112. Jang, S., Uematsu, S., Akira, S. & Salgame, P. (2004) IL-6 and IL-10 induction from dendritic cells in response to Mycobacterium tuberculosis is predominantly dependent on

TLR2-mediated recognition, *J Immunol*. **173**, 3392-7.

113. Offermann, M. K., Zimring, J., Mellits, K. H., Hagan, M. K., Shaw, R., Medford, R. M., Mathews, M. B., Goodbourn, S. & Jagus, R. (1995) Activation of the double-stranded-RNA-activated protein kinase and induction of vascular cell adhesion molecule-1 by poly (I).poly (C) in endothelial cells, *Eur J Biochem*. **232**, 28-36.
114. Lundberg, A. M., Drexler, S. K., Monaco, C., Williams, L. M., Sacre, S. M., Feldmann, M. & Foxwell, B. M. (2007) Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems, *Blood*. **110**, 3245-52.
115. Kang, J. L., Lee, H. W., Lee, H. S., Pack, I. S., Chong, Y., Castranova, V. & Koh, Y. (2001) Genistein prevents nuclear factor-kappa B activation and acute lung injury induced by lipopolysaccharide, *Am J Respir Crit Care Med*. **164**, 2206-12.
116. Wang, P., Wu, P., Siegel, M. I., Egan, R. W. & Billah, M. M. (1994) IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells, *J Immunol*. **153**, 811-6.
117. Hagerman, A. E. & Butler, L. G. (1981) The specificity of proanthocyanidin-protein interactions, *J Biol Chem*. **256**, 4494-7.
118. Tachibana, H., Koga, K., Fujimura, Y. & Yamada, K. (2004) A receptor for green tea polyphenol EGCG, *Nat Struct Mol Biol*. **11**, 380-1.
119. Taub, D. D. & Oppenheim, J. J. (1994) Chemokines, inflammation and the immune system, *Ther Immunol*. **1**, 229-46.
120. Scheerens, H., Hessel, E., de Waal-Malefyt, R., Leach, M. W. & Rennick, D. (2001) Characterization of chemokines and chemokine receptors in two murine models of inflammatory bowel disease: IL-10-/- mice and Rag-2-/- mice reconstituted with CD4+CD45RBhigh T cells, *Eur J Immunol*. **31**, 1465-74.
121. Pulido, R., Bravo, L. & Saura-Calixto, F. (2000) Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay, *J Agric Food Chem*. **48**, 3396-402.
122. Higdon, J. V. & Frei, B. (2003) Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions, *Crit Rev Food Sci Nutr.* **43**, 89-143.
123. Scalbert, A., Johnson, I. T. & Saltmarsh, M. (2005) Polyphenols: antioxidants and beyond, *Am J Clin Nutr.* **81**, 215S-217S.
124. Kausar, H., Jeyabalan, J., Aqil, F., Chabba, D., Sidana, J., Singh, I. P. & Gupta, R. C. (2012) Berry anthocyanidins synergistically suppress growth and invasive potential of human non-small-cell lung cancer cells, *Cancer Lett*. **325**, 54-62.
125. Shi, J., Liu, F., Zhang, W., Liu, X., Lin, B. & Tang, X. (2015) Epigallocatechin-3-gallate inhibits nicotine-induced migration and invasion by the suppression of angiogenesis and

epithelial-mesenchymal transition in non-small cell lung cancer cells, *Oncol Rep*. **33**, 2972-80.

126. Rigalli, J. P., Tocchetti, G. N., Arana, M. R., Villanueva, S. S., Catania, V. A., Theile, D., Ruiz, M. L. & Weiss, J. (2016) The phytoestrogen genistein enhances multidrug resistance in breast cancer cell lines by translational regulation of ABC transporters, *Cancer Lett*. **376**, 165-72.
127. Wang, H., Zhang, H., Tang, L., Chen, H., Wu, C., Zhao, M., Yang, Y., Chen, X. & Liu, G. (2013) Resveratrol inhibits TGF- β 1-induced epithelial-to-mesenchymal transition and suppresses lung cancer invasion and metastasis, *Toxicology*. **303**, 139-46.
128. Tipoe, G. L., Leung, T. M., Hung, M. W. & Fung, M. L. (2007) Green tea polyphenols as an anti-oxidant and anti-inflammatory agent for cardiovascular protection, *Cardiovasc Hematol Disord Drug Targets*. **7**, 135-44.
129. Vita, J. A. (2005) Polyphenols and cardiovascular disease: effects on endothelial and platelet function, *Am J Clin Nutr*. **81**, 292S-297S.
130. Manach, C., Mazur, A. & Scalbert, A. (2005) Polyphenols and prevention of cardiovascular diseases, *Curr Opin Lipidol*. **16**, 77-84.
131. Rathee, P., Chaudhary, H., Rathee, S., Rathee, D., Kumar, V. & Kohli, K. (2009) Mechanism of action of flavonoids as anti-inflammatory agents: a review, *Inflamm Allergy Drug Targets*. **8**, 229-35.
132. Yoon, J. H. & Baek, S. J. (2005) Molecular targets of dietary polyphenols with anti-inflammatory properties, *Yonsei Med J*. **46**, 585-96.
133. Biesalski, H. K. (2007) Polyphenols and inflammation: basic interactions, *Curr Opin Clin Nutr Metab Care*. **10**, 724-8.
134. Mendoza-Wilson, A. M., Castro-Arredondo, S. I., Espinosa-Plascencia, A., Robles-Burgueño, M. e. R., Balandrán-Quintana, R. R. & Bermúdez-Almada, M. e. C. (2016) Chemical composition and antioxidant-prooxidant potential of a polyphenolic extract and a proanthocyanidin-rich fraction of apple skin, *Heliyon*. **2**, e00073.
135. Pearson, D. A., Tan, C. H., German, J. B., Davis, P. A. & Gershwin, M. E. (1999) Apple juice inhibits human low density lipoprotein oxidation, *Life Sci*. **64**, 1913-20.
136. Mueller, D., Triebel, S., Rudakovski, O. & Richling, E. (2013) Influence of triterpenoids present in apple peel on inflammatory gene expression associated with inflammatory bowel disease (IBD), *Food Chem*. **139**, 339-46.
137. McCormick, C. & Ganem, D. (2005) The kaposin B protein of KSHV activates the p38/MK2 pathway and stabilizes cytokine mRNAs, *Science*. **307**, 739-41.
138. Pisegna, S., Pirozzi, G., Piccoli, M., Frati, L., Santoni, A. & Palmieri, G. (2004) p38 MAPK activation controls the TLR3-mediated up-regulation of cytotoxicity and cytokine

production in human NK cells, *Blood*. **104**, 4157-64.

139. Geraldes, P., Hiraoka-Yamamoto, J., Matsumoto, M., Clermont, A., Leitges, M., Marette, A., Aiello, L. P., Kern, T. S. & King, G. L. (2009) Activation of PKC-delta and SHP-1 by hyperglycemia causes vascular cell apoptosis and diabetic retinopathy, *Nat Med*. **15**, 1298-306.
140. Kumar, A., Zhang, J. & Yu, F. S. (2006) Toll-like receptor 3 agonist poly(I:C)-induced antiviral response in human corneal epithelial cells, *Immunology*. **117**, 11-21.
141. Ritter, M., Mennerich, D., Weith, A. & Seither, P. (2005) Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response, *J Inflamm (Lond)*. **2**, 16.
142. Jung, M., Triebel, S., Anke, T., Richling, E. & Erkel, G. (2009) Influence of apple polyphenols on inflammatory gene expression, *Mol Nutr Food Res*. **53**, 1263-80.
143. Akira, S. & Takeda, K. (2004) Toll-like receptor signalling, *Nat Rev Immunol*. **4**, 499-511.
144. Ohmori, Y. & Hamilton, T. A. (1995) The interferon-stimulated response element and a kappa B site mediate synergistic induction of murine IP-10 gene transcription by IFN-gamma and TNF-alpha, *J Immunol*. **154**, 5235-44.
145. Borgland, S. L., Bowen, G. P., Wong, N. C., Libermann, T. A. & Muruve, D. A. (2000) Adenovirus vector-induced expression of the C-X-C chemokine IP-10 is mediated through capsid-dependent activation of NF-kappaB, *J Virol*. **74**, 3941-7.
146. Brownell, J., Bruckner, J., Wagoner, J., Thomas, E., Loo, Y. M., Gale, M., Liang, T. J. & Polyak, S. J. (2014) Direct, interferon-independent activation of the CXCL10 promoter by NF- κ B and interferon regulatory factor 3 during hepatitis C virus infection, *J Virol*. **88**, 1582-90.
147. Lu, X., Masic, A., Liu, Q. & Zhou, Y. (2011) Regulation of influenza A virus induced CXCL-10 gene expression requires PI3K/Akt pathway and IRF3 transcription factor, *Mol Immunol*. **48**, 1417-23.
148. Schmitz, F., Mages, J., Heit, A., Lang, R. & Wagner, H. (2004) Transcriptional activation induced in macrophages by Toll-like receptor (TLR) ligands: from expression profiling to a model of TLR signaling, *Eur J Immunol*. **34**, 2863-73.
149. Booth, A. D., Wallace, S., McEniery, C. M., Yasmin, Brown, J., Jayne, D. R. & Wilkinson, I. B. (2004) Inflammation and arterial stiffness in systemic vasculitis: a model of vascular inflammation, *Arthritis Rheum*. **50**, 581-8.
150. Kals, J., Kampus, P., Kals, M., Pulges, A., Teesalu, R., Zilmer, K., Kullisaar, T., Salum, T., Eha, J. & Zilmer, M. (2008) Inflammation and oxidative stress are associated differently with endothelial function and arterial stiffness in healthy subjects and in patients with

atherosclerosis, *Scand J Clin Lab Invest*. **68**, 594-601.

151. Kals, J., Kampus, P., Kals, M., Zilmer, K., Kullisaar, T., Teesalu, R., Pulges, A. & Zilmer, M. (2006) Impact of oxidative stress on arterial elasticity in patients with atherosclerosis, *Am J Hypertens*. **19**, 902-8.
152. Alzoreky, N. S. & Nakahara, K. (2003) Antibacterial activity of extracts from some edible plants commonly consumed in Asia, *Int J Food Microbiol*. **80**, 223-30.
153. Rosenblat, M., Hayek, T. & Aviram, M. (2006) Anti-oxidative effects of pomegranate juice (PJ) consumption by diabetic patients on serum and on macrophages, *Atherosclerosis*. **187**, 363-71.
154. Zahin, M., Aqil, F. & Ahmad, I. (2010) Broad spectrum antimutagenic activity of antioxidant active fraction of punica granatum L. peel extracts, *Mutat Res*. **703**, 99-107.
155. Singh, R. P., Chidambara Murthy, K. N. & Jayaprakasha, G. K. (2002) Studies on the antioxidant activity of pomegranate (Punica granatum) peel and seed extracts using in vitro models, *J Agric Food Chem*. **50**, 81-6.
156. Parmar, H. S. & Kar, A. (2007) Antidiabetic potential of Citrus sinensis and Punica granatum peel extracts in alloxan treated male mice, *Biofactors*. **31**, 17-24.
157. Cohen, R. D. (2010) The pharmacoeconomics of biologic therapy for IBD, *Nat Rev Gastroenterol Hepatol*. **7**, 103-9.
158. Kaser, A. & Blumberg, R. S. (2008) Adaptive immunity in inflammatory bowel disease: state of the art, *Curr Opin Gastroenterol*. **24**, 455-61.
159. McCarthy, D. A., Rampton, D. S. & Liu, Y. C. (1991) Peripheral blood neutrophils in inflammatory bowel disease: morphological evidence of in vivo activation in active disease, *Clin Exp Immunol*. **86**, 489-93.
160. Mee, A. S., Berney, J. & Jewell, D. P. (1980) Monocytes in inflammatory bowel disease: absolute monocyte counts, *J Clin Pathol*. **33**, 917-20.
161. Boirivant, M., Marini, M., Di Felice, G., Pronio, A. M., Montesani, C., Tersigni, R. & Strober, W. (1999) Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis, *Gastroenterology*. **116**, 557-65.
162. Saubermann, L. J., Probert, C. S., Christ, A. D., Chott, A., Turner, J. R., Stevens, A. C., Balk, S. P. & Blumberg, R. S. (1999) Evidence of T cell receptor beta-chain patterns in inflammatory and noninflammatory bowel disease states, *Am J Physiol*. **276**, G613-21.
163. Zareie, M., Singh, P. K., Irvine, E. J., Sherman, P. M., McKay, D. M. & Perdue, M. H. (2001) Monocyte/macrophage activation by normal bacteria and bacterial products: implications for altered epithelial function in Crohn's disease, *Am J Pathol*. **158**, 1101-9.
164. Taub, D. D., Lloyd, A. R., Conlon, K., Wang, J. M., Ortaldo, J. R., Harada, A., Matsushima, K., Kelvin, D. J. & Oppenheim, J. J. (1993) Recombinant human interferon-inducible

protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells, *J Exp Med*. **177**, 1809-14.

165. Wang, L., Alcon, A., Yuan, H., Ho, J., Li, Q. J. & Martins-Green, M. (2011) Cellular and molecular mechanisms of pomegranate juice-induced anti-metastatic effect on prostate cancer cells, *Integr Biol (Camb)*. **3**, 742-54.
166. Afaq, F., Malik, A., Syed, D., Maes, D., Matsui, M. S. & Mukhtar, H. (2005) Pomegranate fruit extract modulates UV-B-mediated phosphorylation of mitogen-activated protein kinases and activation of nuclear factor kappa B in normal human epidermal keratinocytes paragraph sign, *Photochem Photobiol*. **81**, 38-45.
167. Schubert, S. Y., Neeman, I. & Resnick, N. (2002) A novel mechanism for the inhibition of NF-kappaB activation in vascular endothelial cells by natural antioxidants, *FASEB J*. **16**, 1931-3.
168. Afaq, F., Saleem, M., Krueger, C. G., Reed, J. D. & Mukhtar, H. (2005) Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF-kappaB pathways and inhibits skin tumorigenesis in CD-1 mice, *Int J Cancer*. **113**, 423-33.
169. Watanabe, H., Numata, K., Ito, T., Takagi, K. & Matsukawa, A. (2004) Innate immune response in Th1- and Th2-dominant mouse strains, *Shock*. **22**, 460-6.
170. Petrai, I., Rombouts, K., Lasagni, L., Annunziato, F., Cosmi, L., Romanelli, R. G., Sagrinati, C., Mazzinghi, B., Pinzani, M., Romagnani, S., Romagnani, P. & Marra, F. (2008) Activation of p38(MAPK) mediates the angiostatic effect of the chemokine receptor CXCR3-B, *Int J Biochem Cell Biol*. **40**, 1764-74.
171. Alrashdan, Y. A., Alkhouri, H., Chen, E., Lalor, D. J., Poniris, M., Henness, S., Brightling, C. E., Burgess, J. K., Armour, C. L., Ammit, A. J. & Hughes, J. M. (2012) Asthmatic airway smooth muscle CXCL10 production: mitogen-activated protein kinase JNK involvement, *Am J Physiol Lung Cell Mol Physiol*. **302**, L1118-27.
172. Marín, M., María Giner, R., Ríos, J. L. & Recio, M. C. (2013) Intestinal anti-inflammatory activity of ellagic acid in the acute and chronic dextrane sulfate sodium models of mice colitis, *J Ethnopharmacol*. **150**, 925-34.
173. Kim, Y. H., Ninomiya, Y., Yamashita, S., Kumazoe, M., Huang, Y., Nakahara, K., Won, Y. S., Murata, M., Fujimura, Y., Yamada, K. & Tachibana, H. (2014) IL-4 receptor α in non-lipid rafts is the target molecule of strictinin in inhibiting STAT6 activation, *Biochem Biophys Res Commun*. **450**, 824-30.
174. Toklu, H. Z., Dumlu, M. U., Sehirli, O., Ercan, F., Gedik, N., Gökmen, V. & Sener, G. (2007) Pomegranate peel extract prevents liver fibrosis in biliary-obstructed rats, *J Pharm Pharmacol*. **59**, 1287-95.
175. Lin, C. C., Hsu, Y. F. & Lin, T. C. (1999) Effects of punicalagin and punicalin on

carrageenan-induced inflammation in rats, *Am J Chin Med*. **27**, 371-6.

176. Majumder, S., Zhou, L. Z., Chaturvedi, P., Babcock, G., Aras, S. & Ransohoff, R. M. (1998) Regulation of human IP-10 gene expression in astrocytoma cells by inflammatory cytokines, *J Neurosci Res*. **54**, 169-80.

177. Mehla, R., Bivalkar-Mehla, S., Nagarkatti, M. & Chauhan, A. (2012) Programming of neurotoxic cofactor CXCL-10 in HIV-1-associated dementia: abrogation of CXCL-10-induced neuro-glial toxicity in vitro by PKC activator, *J Neuroinflammation*. **9**, 239.

178. Yamauchi, N., Harada, T., Taniguchi, F., Yoshida, S., Iwabe, T. & Terakawa, N. (2004) Tumor necrosis factor-alpha induced the release of interleukin-6 from endometriotic stromal cells by the nuclear factor-kappaB and mitogen-activated protein kinase pathways, *Fertil Steril*. **82 Suppl 3**, 1023-8.

179. Németh, Z. H., Deitch, E. A., Szabó, C. & Haskó, G. (2003) Pyrrolidinedithiocarbamate inhibits NF-kappaB activation and IL-8 production in intestinal epithelial cells, *Immunol Lett*. **85**, 41-6.

180. Cooray, S. (2004) The pivotal role of phosphatidylinositol 3-kinase-Akt signal transduction in virus survival, *J Gen Virol*. **85**, 1065-76.

181. Liu, Q., White, L. R., Clark, S. A., Heffner, D. J., Winston, B. W., Tibbles, L. A. & Muruve, D. A. (2005) Akt/protein kinase B activation by adenovirus vectors contributes to NFkappaB-dependent CXCL10 expression, *J Virol*. **79**, 14507-15.

182. Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakanishi, T., Nakae, H. & Matsuo, T. (2010) Tea polyphenols inhibit IL-6 production in tumor necrosis factor superfamily 14-stimulated human gingival fibroblasts, *Mol Nutr Food Res*. **54 Suppl 2**, S151-8.

183. Cai, Y. Z., Mei Sun, Jie Xing, Luo, Q. & Corke, H. (2006) Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants, *Life Sci*. **78**, 2872-88.

184. McNabb, W. C., Waghorn, G. C., Barry, T. N. & Shelton, I. D. (1993) The effect of condensed tannins in Lotus pedunculatus on the digestion and metabolism of methionine, cystine and inorganic sulphur in sheep, *Br J Nutr.* **70**, 647-61.

185. Tachibana, H. (2008) Green tea polyphenol EGCG signaling pathway through the 67-kDa laminin receptor, *Nihon Yakurigaku Zasshi*. **132**, 145-9.

186. Adisakwattana, S., Jiphimai, P., Prutanopajai, P., Chanathong, B., Sapwarobol, S. & Ariyapitipan, T. (2010) Evaluation of alpha-glucosidase, alpha-amylase and protein glycation inhibitory activities of edible plants, *Int J Food Sci Nutr.* **61**, 295-305.

187. Xu, X., Yin, P., Wan, C., Chong, X., Liu, M., Cheng, P., Chen, J., Liu, F. & Xu, J. (2014) Punicalagin inhibits inflammation in LPS-induced RAW264.7 macrophages via the

suppression of TLR4-mediated MAPKs and NF- κ B activation, *Inflammation*. **37**, 956-65.

188. Xu, X., Li, H., Hou, X., Li, D., He, S., Wan, C., Yin, P., Liu, M., Liu, F. & Xu, J. (2015) Punicalagin Induces Nrf2/HO-1 Expression via Upregulation of PI3K/AKT Pathway and Inhibits LPS-Induced Oxidative Stress in RAW264.7 Macrophages, *Mediators Inflamm*. **2015**, 380218.

189. Olajide, O. A., Kumar, A., Velagapudi, R., Okorji, U. P. & Fiebich, B. L. (2014) Punicalagin inhibits neuroinflammation in LPS-activated rat primary microglia, *Mol Nutr Food Res*. **58**, 1843-51.

Appendices

Manuscript I:

Polymerization degree-dependent inhibitory effect of apple condensed tannin on interleukin-6 expression of intestinal epithelial cells.
Authors: Peng Li, Yasuharu Ishihara, Ayako Aoki-Yoshida, Makoto Shimizu, Mamoru Totsuka*
(In preparation, 2017/3)

Manuscript II:

Apple condensed tannins regulate chemokines CXCL10 expression via activation of MAPKs signaling pathway in intestinal epithelial cells.
Authors: Peng Li, Makoto Shimizu, Ayako Aoki-Yoshida, Mamoru Totsuka*
(In preparation, 2017/3)

Manuscript III:

Pomegranate peel-derived polyphenols stimulates CXCL10 genes expression via activation of PKC, MEK1/2, ERK1/2, JNK, and PI3K-IRF3 signaling pathways in intestinal epithelial cells.
Authors: Peng Li, Hideyuki Ito, Ayako Aoki-Yoshida, Mamoru Totsuka*
(In preparation, 2017/3)

Conferences and Poster Presentation

1　李鵬、青木　綾子、戸塚　護

ポリフェノール類が腸管上皮細胞によるケモカインの産生を誘導する効果に関する研究

日本農芸化学会 2015 年度大会、岡山、2015 年 3 月

2　李　鵬、青木　綾子、戸塚　護

ポリフェノール類が腸管上皮細胞によるケモカインの産生を誘導するメカニズムの解明に関する研究

第 28 回日本動物細胞工学会、仙台、2015 年 7 月

3　李　鵬、青木　綾子、伊東　秀之、戸塚　護

Effect of extracts from different parts of pomegranate on chemokines production by intestinal epithelial cells

日本農芸化学会 2016 年度大会、札幌、2016 年 3 月

Abstract

Introduction

Polyphenols, one of the most common groups of plant secondary metabolites, have received considerable interest over the past few years due to their presumed role in the prevention of various degenerative diseases. Apple (*Malus pumila*) is widely consumed, and is rich source of polyphenols. Apple components investigation showed that apple peel contains an abundance of procyanidins and their polymers (apple condensed tannins; ACT). Accumulated evidence indicates that the consumption of apple and apple products could reduce risks of cardiovascular diseases, cancers, asthma, diabetes, obesity, and pulmonary dysfunction. These pharmacological activities from apple and apple-processed products have been shown to be associated with ACT, which abound in unripe apple peel more than ripe one.

Pomegranate (*Punica granatum* L.) fruit is widely consumed fresh and in processed forms as juice, jam, and wine. Pomegranate peels and leaves were used in the traditional medicine of different Asian cultures for the treatment of a variety of ailments. Phytochemical studies previously have revealed that ellagitannins (ETs) and anthocyanins (ANs) represent the most abundant polyphenols in pomegranate aril and peel. Hydrolysable tannin, strictinin is the main phenolic component present in pomegranate leaves. Importantly, numerous scientific evidence demonstrates that the health benefits of pomegranate and their products have been positively associated with the polyphenols in these fruits.

It is well known that cytokines play a key role in the intestinal immune system. Inflammatory bowel diseases (IBD) are chronic inflammatory diseases of the gastrointestinal tract. Inflammatory cytokine interleukin 6 (IL-6) and chemokine CXCL10 have been identified as important cytokines in IBD. IL-6 is a pleiotropic cytokine that exerts its proinflammatory effects largely by means of its soluble IL-6 receptor. It has been reported that altered IL-6 production has been found in IBD affected patients. Chemokines CXCL9, CXCL10, and CXCL11 are known to activate a common receptor CXCR3, which is commonly expressed on activated T cells, B cells, and NK cells. These three chemokines recruit activated T cells into the intestinal mucosa. In addition, high levels of CXCL10 release have also been found in patients with IBDs. On the other hand, these cytokines are important in activation of the immune system to prevent infection in the intestine at non-inflammatory steady state.

Recently, numerous scientific evidence suggested that polyphenols appear as promising candidates for up-regulating or down-regulating cytokines expression. However, the

influences of apple peel-derived polyphenols and pomegranate-derived polyphenols on IL-6 and CXCL10 expression in intestinal epithelial cells have not yet been revealed. The present study aimed to investigate the effects of ACT and pomegranate aril extract, peel extract, and leaf extract on the IL-6 and CXCL10 expression in intestinal epithelial cells, and to examine the effects of apple extracts and pomegranate extracts on cell signaling pathways to uncover the mechanism.

Chapter 1. Polymerization degree-dependent inhibitory effect of apple condensed tannins on IL-6 production in mouse intestinal epithelial cells

Poly I:C is structurally similar to dsRNA as a respective agonist for toll-like receptor 3 (TLR3) which selectively triggers TLR3-dependent up-regulation of IL-6 and CXCL10 production. This chapter describes the effects of ACT on Poly I:C-induced IL-6 expression in mouse small intestinal epithelial cell line, MoS13 cells. Since ACT is a complex flavonoid polymer, consist of oligomeric (-)-epicatechin of 2-14 mers, the effect of smaller fractions of ACT (2-mer) and larger fractions of ACT (7-mer) on IL-6 expression was also investigated.

ACT at 25, 50 μg/mL was found to inhibit Poly I:C-mediated IL-6 mRNA expression, transcription and protein expression by MoS13 cells. In addition, I evaluated the effect of ACT on signaling pathways by western blot analysis. This effect was found to be regulated by p38, MEK1/2, JNK-MAPKs, and IκB pathways. However, the smaller fraction of ACT (monomer and dimer) had no effect on the release of IL-6 from Poly I:C-induced MoS13 cells. Whereas Poly I:C-mediated IL-6 production and mRNA expression by the cells was robustly inhibited by larger fractions of ACT (7-mer). These results suggested that higher concentration of ACT and larger fractions of ACT down-regulated Poly I:C-induced IL-6 expression polymerization-degree dependently by suppressing NF-κB and MEK1/2-, JNK activity.

Chapter 2. Apple polyphenols regulate chemokines CXCL9, CXCL10, and CXCL11 expression in intestinal epithelial cells via activation of MAPKs pathway

In Chapter 2, I investigated the effect of ACT on Poly I:C-induced expression of chemokines CXCL9, CXCL10 and CXCL11 by the intestinal epithelial cells. MoS13 cells were treated with ACT at four concentrations (0.7, 3.1, 12.5, 50 μg/mL) in the presence of Poly I:C. To my surprise, the data demonstrated that ACT stimuli at 3.1 or 12.5 μg/mL significantly up-regulated Poly I:C-induced CXCL9, CXCL10, and CXCL11 expression. Interestingly, ACT (12.5 μg/mL) did not up-regulate Pam3CSK3 (TLR1/2 ligand)- and LPS (TLR4 ligand)-induced expression of these chemokines. In addition, ELISA and luciferase reporter gene assay analysis

revealed that ACT (12.5 μg/mL) significantly increased the production of CXCL10 and CXCL11 in Poly I:C-stimulated MoS13 cells and increased promoter activity of the CXCL10 gene as well. Furthermore, the analysis using inhibitors of signaling pathways suggested that suppression of PKC, MEK1/2, JNK and p38 pathways with their specific inhibitors significantly prevented the up-regulation of CXCL10 gene expression. Subsequently, the effect of ACT on Poly I:C-induced signaling pathway was evaluated by western blot analysis. It was confirmed that ACT enhanced the phosphorylation of MEK, JNK, and p38, and the nuclear translocation of IRF3, whereas ACT stimuli did not increase the phosphorylation of IκB. In *in vivo* experiments, an increased CXCL10 expression was observed in the intestine of BALB/c mice receiving ACT by oral administration.

In conclusion, these results in Chapter 1 and Chapter 2 suggest that ACT at different concentrations may show a different regulating effect on IL-6 and CXCL10 expression. ACT (12.5 μg/mL) up-regulated Poly I:C-induced CXCL10 gene expression predominantly via enhancing PI3K-IRF3 signaling pathways, but not via IκB signaling pathway. On the other hand, ACT (50 μg/mL) down-regulated Poly I:C induced IL-6 gene expression predominantly via suppressing IκB and MEK1/2, JNK, p38 signaling pathways.

Chapter 3. Pomegranate peel-derived polyphenols stimulate CXCL10 gene expression and production via activation of PKC-MAPKs and PI3K-IRF3 signaling pathways in intestinal epithelial cells

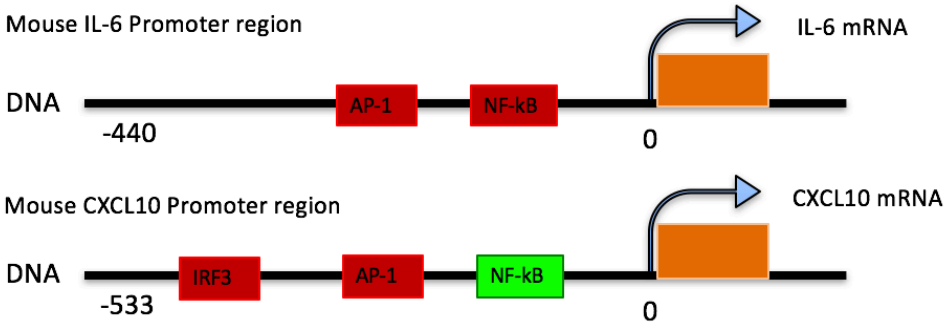
The aim of the study in Chapter 3 was to investigate the effects of pomegranate aril extract (PAE), pomegranate peel extract (PPE), pomegranate leaf extract (PLE) on chemokines expression in the intestine *in vivo* and *in vitro*. BALB/c mice were fed a normal diet and either water or water containing 1% PAE, 1% PPE, and 1% PLE ad libitum over a period of two weeks. Interestingly, only oral administration of 1% PPE significantly up-regulated CXCL9 and CXCL10 gene expression in the intestinal mucosa. In addition, we examined the enhancement effect of PPE on the chemokine gene expression *in vitro* using MoS13 cells. I found that treatment of MoS13 cells with PPE at a concentration of 50 μg/mL for 20 h, significantly increased CXCL9, CXCL10, and CXCL11 gene expression. It was observed that PPE stimulation significantly enhanced the production of CXCL10 and the transcriptional activation of CXCL10 gene as well. On the transcriptional level, CXCL10 promoter activation was known to be regulated by NF-κB and IRF3 binding sites, and by upstream MAPKs signaling pathways.

To reveal the mechanism, first, we evaluated the effect of each inhibitor on respective signaling pathways. We observed that the induction of CXCL10 gene expression was

suppressed by pharmacological inhibitors of PKC (RO31-8220), PI3K (LY294002), MEK1/2-ERK (PD98059, U0126), P38 (SB203580), or JNK (SP600125), suggesting the involvement of PKC and MAPKs pathways in CXCL10 gene expression. In contrast, NF-κB inhibitor did not suppress PPE-induced CXCL10 gene expression. Importantly, the western blot analysis results suggested that PPE stimulation significantly increased the phosphorylation of MEK1/2, ERK1/2, JNK, but did not affect the phosphorylation of p38 and IκB. Furthermore, we observed that PPE treatment significantly increased the nuclear translocation of IRF3, while did not affect the nuclear translocation of NF-κB. In conclusion, this study suggests that PPE enhanced CXCL10 expression in intestinal mucosa via activation of PKC, MEK1/2, ERK1/2, and JNK signaling pathways and PI3K-IRF3 pathway.

Conclusions

In this whole works, I have investigated the effect of ACT on IL-6 and CXCL10 gene expression, and also compared the effect of PAE, PPE, and PLE on CXCL10 gene expression in mouse intestinal epithelial cells. It was observed that ACT showed different regulating effect on IL-6 and CXCL10 gene expression in Poly I:C-induced MoS13 cells. Here, I suggest that higher concentration of ACT (50 μg/mL) inhibit IL-6 expression predominantly via suppressing IκB signaling pathway, and PKC-MAPKs (MEK1/2, JNK, p38) signaling pathways. In contrast, in Chapter 2, I first found that lower concentration of ACT (12.5 μg/mL) up-regulated Poly I:C-induced CXCL10 expression predominantly via activation of PI3K-IRF3 signaling pathways, but not via IκB signaling pathway. In Chapter 3, PPE was found to up-regulate CXCL10 expression predominantly via activation of PI3K-IRF3 signaling pathway and PKC-MAPKs (MEK1/2, ERK1/2, and JNK) signaling pathway. Whereas PPE did not induce IκB signaling pathway. On the transcriptional level, there is no binding site for IRF3 in the IL-6 gene promoter region, while IRF3 play a central role in CXCL10 gene expression. These results indicate that ACT and PPE may predominantly up-regulate PI3K-IRF3 signaling pathway, which is involved in the increase of CXCL10 expression, while ACT may predominantly down-regulate IκB signaling pathway, resulting in the decrease of IL-6 expression. These findings suggest that apple peel extract and pomegranate peel extract may regulate cytokine and chemokine expression in intestinal epithelial cells via different signaling pathways. These results also support that consumption of apples and pomegranate may help protect against intestine disorder by decreasing pro-inflammatory cytokines and enhancing gut immunity with increasing chemokines in the normal steady state.


Mouse IL-6 Promoter region
IL-6 mRNA
DNA
AP-1
NF-kB
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Mouse CXCL10 Promoter region
CXCL10 mRNA
DNA
IRF3
AP-1
NF-kB
-533
0

論文の内容の要旨

論文題目

Effect of apple-derived condensed tannins and pomegranate-derived polyphenols on cytokine expression of intestinal epithelial cells

（リンゴ由来縮合タンニンとザクロ由来ポリフェノールが腸管上皮細胞の
サイトカイン応答に与える影響に関する研究）

研究の背景と目的

ポリフェノール類は植物の生産する二次代謝産物のうちの一つであり、抗酸化作用、抗炎症作用などの有用な生理活性が数多く見いだされているため、近年医学、栄養学、食品化学、生薬学などさまざまな分野で注目を帯びてきた。リンゴは世界的に広く摂取されている果物でポリフェノールを豊富に含み、一日 1 個リンゴで医者いらずということわざもある。リンゴの成分分析によると、リンゴ果皮、特に未熟なリンゴ果皮にはカテキンあるいはエピカテキンで構成されるプロシアニジン類とその重合体（リンゴ縮合タンニン; ACT）が多く含まれている。リンゴおよびリンゴ加工品は心血管疾患、がん、喘息、糖尿病、肥満、肺機能障害などの疾患を予防する効果を有していることが明らかとなっている。これまでの数多くの研究から、ACT はリンゴの機能性に最も寄与しており、日々の健康維持に重要な役割を果たしていると考えられる。

ザクロはトルコ、イラン、インドおよびヨーロッパなどでよく食べられている果物であり、生食、あるいはジュースや、ジャム、ワインなどのかたちでよく摂取されている。ザクロ果皮とザクロ葉は古くからアジアの様々な地域において伝統的な薬として様々な疾患の治療のために用いられてきた。ザクロの植物化学成分の分析により、エラジタンニン（ETs）およびアントシアニン（ANs）が、果肉と果皮に最も多く存在するポリフェノールであることが示されている。加水分解型タンニンであるストリクチニンは葉の主要なフェノール性成分である。ザクロおよびその加工品の多様な生理活性には、ザクロに含まれるポリフェノール類が寄与していることがこれまでの多くの研究成果により示されている。

サイトカインは腸管免疫システムにおいて鍵となる働きをしていることはよく知られている。炎症性腸疾患（IBD）は消化管の慢性的炎症による疾患である。炎症性サイトカイン IL-6 およびケモカイン CXCL10 は IBD の病態において重要なファクターだと考えている。IL-6 は様々な機能を有するサイトカインであり、IL-6 受容体との反

応により炎症を惹起する。また、IBD の患者から高い IL-6 産生量を検出する報告もある。CXCL9, CXCL10, および CXCL11 は T 細胞、B 細胞、および NK 細胞などの免疫細胞表面の共通リセプターCXCR3 と結合することにより免疫細胞の動員を促進することで知られている。これらケモカインは主に炎症時に活性化された T 細胞の遊走を促進することが報告された。IBD の患者から、高いケモカイン CXCL10 産生も確認されている。一方、これらサイトカインおよびケモカインは定常状態においては、感染を防ぐために腸管免疫系の活性化に対して重要なフェクターでもある。

近年、天然物由来ポリフェノールはこれらのサイトカインやケモカインの産生を増強あるいは低下させることが示唆されている。しかしながら、リンゴ由来ポリフェノールおよびザクロ由来ポリフェノールは、腸管上皮細胞における IL-6 および CXCL10 の産生に対する影響は未だ不明な点が多い。そこで本研究では、リンゴ果皮由来の縮合タンニン（ACT）、ザクロ果肉由来ポリフェノール（PAE）、ザクロ果皮由来ポリフェノール（PPE）、ザクロ葉由来ポリフェノール（PLE）を用いて、腸管上皮細胞における IL-6, CXCL10 の産生に対する影響を解明すること、さらに、シグナリング経路に対する影響を解析し作用機作を明らかにすることを目的とした。

第 1 章　腸管上皮細胞における IL-6 産生に対するリンゴ縮合タンニンの重合度依存的な抑制作用

Poly I:C はウイルスの dsRNA と同様の構造をもち Toll 様受容体 3（TLR3）のアゴニストである。Poly I:C は TLR3 依存的に腸管上皮細胞を刺激し、IL-6 や CXCL10 の産生を誘導する。本章では、まず、当研究室で樹立されたマウス小腸上皮細胞株 MoS13 細胞を Poly I:C 刺激して誘導される IL-6 産生に対する ACT の影響を調べた。ACT は一つの化合物ではなく、(-)-エピカテキンの 2 量体から 14 量体程度までの混合物であるため、 低重合度 ACT(2-mer)および高重合度 ACT (7-mer)が腸管上皮細胞における IL-6 産生に与える影響も調べた。

Poly I:C によって誘導された IL-6 の mRNA 発現、タンパク質発現および転写活性は ACT（25, 50 μg/mL）によって強く抑制されることが明らかとなった。さらに、ACT がシグナル伝達経路に与える影響をウエスタンブロット法により検討した。その結果、Poly I:C によって増加した p38, MEK1/2, JNK および IκB,のリン酸化に対し、ACT（50 μg/mL）が強い抑制作用を示したことから、これらのシグナル経路の関与が示された。また、Poly I:C によって誘導された IL-6 産生及び mRNA 発現は ACT (7-mer)によって強く抑制されたのに対し、ACT(2-mer)は IL-6 産生を抑制しなかった。以上の結果から、高重合度の ACT は NF-κB、MEK1/2-MAPK および JNK-MAPK 経路を抑制すること

によって、Poly I:C によって誘導された IL-6 産生を抑制することが示唆された。

第 2 章　腸管上皮細胞における CXCL9, CXCL10 と CXCL11 産生に対するリンゴ縮合タンニンの誘導作用

本章では、腸管上皮細胞における Poly I:C 刺激で誘導されたケモカイン CXCL9, CXCL10 および CXCL11 の産生に対する ACT の影響を調べた。MoS13 細胞に 4 段階の濃度の ACT を Poly I:C 刺激下で添加し、5 時間後のケモカインの発現を測定した。驚いたことに、Poly I:C によって誘導された CXCL9, CXCL10 および CXCL11 の発現に対して、濃度 3.1 μg/mL と 12.5 μg/mL の ACT は有意な亢進作用が確認された。興味深いことに、ACT は TLR1/2 のリガンドである Pam3CSK4 あるいは TLR4 のリガンドである LPS によって誘導された CXCL9, CXCL10 および CXCL11 の発現に対しては増強効果を示さなかった。また、ACT は Poly I:C 刺激で誘導された CXCL10 と CXCL11 のタンパク質量および CXCL10 の転写活性を有意に亢進した。さらに、ACT がどのようなシグナル伝達経路を介して CXCL10 産生を亢進しているかについて明らかにするために、様々なシグナル伝達経路阻害剤を用いて検討した。その結果、PKC 阻害剤（RO31-8220）, MEL1/2 阻害剤（PD98059）, JNK 阻害剤（SP600125）, p38 阻害剤（SB203580）の添加によって、ACT により誘導された CXCL10 発現量増加が抑制された。次に、Poly I:C が誘導するシグナル経路に対する ACT の影響をウエスタンブロット解析で検討したところ、ACT は MEK1/2, JNK, p38 のリン酸化および IRF3 の核内移行を亢進することが確認されたが、IκB のリン酸化に対しては変化が認めらなかった。*In vivo* 実験においても、ACT のマウスに経路投与により、腸管上皮細胞における CXCL10 産生が増加する効果も認められた。

第 1 章と第 2 章の結果により、ACT は濃度の違いによって、腸管上皮細胞における IL-6 と CXCL10 産生に異なる制御効果を示すことが示唆された。低濃度 ACT (12.5 μg/mL)は主に PI3K-IRF3 シグナル伝達経路の活性増強を介して CXCL10 産生を亢進する一方、高濃度 ACT (50 μg/mL)は主に IκB, MEK1/2, JNK および p38 シグナル伝達経路を抑制することで IL-6 産生を抑制することが示唆された。

第 3 章　ザクロ果皮由来ポリフェノールによる腸管上皮細胞における PKC-MAPKs と PI3K-IRF3 シグナル伝達経路を介した CXCL10 産生誘導効果

本章では、ザクロ果肉抽出物（PAE）、ザクロ果皮抽出物（PPE）、ザクロ葉抽出物（PLE）の腸管におけるケモカイン発現に及ぼす影響を、*in vivo* および *in vitro* において調べることを目的とした。BALB/c マウスに通常食とともに、1% PAE、1% PPE、

あるいは 1% PLE を含む水を２週間自由摂取させた。腸管粘膜を回収し、mRNA 発現を解析したところ、興味深いことに 1% PPE を摂取させた場合にのみ、CXCL9 および CXCL10 の遺伝子発現が有意に増加することが初めて明らかとなった。腸管上皮細胞 MoS13 を用いた *in vitro* 実験系においても、PPE が上記ケモカインの遺伝子発現に与える影響を検討した。その結果、濃度 50 μg/mL の PPE 添加により、MoS13 細胞の CXCL9, CXCL10, および CXCL11 の遺伝子発現を強く亢進することが確認できた。さらに、PPE は CXCL10 のタンパク質産生と転写活性を強く亢進したことが認められた。転写レベルにおいては、 CXCL10 の転写活性は CXCL10 遺伝子プロモーター領域にある NF-κB および IRF3 結合サイトおよびそれらの上流シグナルの MAPKs シグナル伝達経路により制御されていることが知られている。

この作用機作を解明するため、まずそれぞれのシグナル伝達経路阻害剤を用いて検討をおこなった。その結果、PKC 阻害剤、PI3K 阻害剤（LY294002）、MEK-ERK 阻害剤（PD98059 および U0126）、p38 阻害剤、 あるいは JNK 阻害剤の添加によって、PPE によって誘導された CXCL10 発現が有意に抑制された。一方、NF-κB 阻害剤（PDTC）は抑制作用を示さなかった。これらの結果から、PPE 刺激は NF-κB のシグナル伝達経路ではなく、PKC, PI3K, MAPKs のシグナル伝達経路を介することが示唆された。さらに、PPE の刺激により MEK, ERK, JNK のリン酸化を強く亢進したことも認められた。一方、p38 と IκB のリン酸化に対しては亢進効果が認められなかった。また、PPE の刺激により IRF3 の核内移行が促進された一方、NF-κB の核移行には影響しないことも明らかとなった。以上の結果から、PPE は腸管上皮細胞において、PKC, MEK, ERK, JNK および PI3K-IRF3 のシグナル伝達経路を介して、CXCL10 発現を亢進することが示唆された。

総括

本研究では、リンゴ由来縮合タンニン(ACT)およびザクロポリフェノール(PAE, PPE, PLE)が腸管上皮細胞における IL-6 および CXCL10 産生に与える影響を解析した。その結果、Poly I:C 刺激を受けた MoS13 細胞において、ACT は IL-6 および CXCL10 の遺伝子発現に対して異なる作用を示すことが明らかとなった。第 1 章では、高濃度の ACT は主に IκB へのシグナル伝達経路の抑制を介して Poly I:C 刺激により誘導された IL-6 産生を抑制しており、PKC-MAPKs (MEK1/2、JNK、p38) シグナル伝達経路も関与することが示唆された。一方、第 2 章では低濃度の ACT による CXCL10 産生亢進には IκB の経路は寄与せず、主に PI3K-IRF3 シグナル伝達経路の活性化を介して Poly I:C 刺激で誘導された CXCL10 産生を亢進することを初めて示した。第 3 章では PPE は

PKC-MAPKs (MEK1/2, ERK1/2, and JNK) および PI3K-IRF3 シグナル伝達経路の活性化を介して、CXCL10 発現を増強することを初めて見出した。またこの時、PPE は NF-κB の活性化には影響を与えていなかった。転写レベルでは、IL-6 遺伝子の発現は転写因子である C/EBP と NF-κB による制御を受けており、プロモーター領域には IRF3 結合サイトは存在しない。一方、CXCL10 の転写活性には主に IRF3 が働いている。これらの知見は、ACT および PPE は腸管上皮細胞において異なるシグナル伝達経路を介してサイトカイン・ケモカイン遺伝子の発現を制御していることを示唆している。本研究は、リンゴ及びザクロの摂取は、炎症性サイトカインの抑制により腸管の疾患から保護することや、健常時においてケモカイン産生増強による腸管免疫応答の増強に寄与することを支持するものである。

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