Drug Innovation Master Thesis

The inhibitory effects of flavonoids on chemokine function in allergic asthma and food allergy

Anna Oja
Student number: 3937488
a.e.oja@students.uu.nl

December 2013 – March 2014

Supervisor/examiner: Dr. Linette Willemsen
Second reviewer: Prof. Dr. Johan Garssen

The inhibitory effects of flavonoids on chemokine function in allergic asthma and food allergy

Anna Oja

Allergic diseases, such as allergic asthma and food allergy, have been burdening people for decades and still no cure exists. The best and only plausible treatment is to avoid the causative allergen. Furthermore, there appears to be an increasing trend in the incidence of allergic diseases in the last few decades, which probably is due to a combination of genetic and environmental factors. Chemokines are small chemotactic cytokines that play crucial roles in the trafficking and recruiting of inflammatory cells in both allergic sensitization and inflammation. Although several research groups and companies have developed antagonists against chemokines and/or the respective chemokine receptors, the success has been limited. Recently, interest in dietary intervention as a therapeutic means has arisen. Flavonoids, bioactive polyphenolic plant secondary metabolites, are being investigated due to their anti-inflammatory, anti-oxidant and immune-modulating traits. Many studies, *in vitro* and *in vivo*, have presented the inhibitory effects of various flavonoids on allergic asthma and a few have done so for food allergy. Thus far, the studies show that flavonoids are able to inhibit chemokine function, but there is still debate over the mechanisms behind these effects. According to some studies, flavonoids suppress the transcription factor, nuclear factor kappa B (NF-κB), but according to other studies flavonoids inhibit various mitogen-activated protein kinase (MAPK) pathways or caspase-1. Flavonoids also exhibit inhibitory effects on dendritic cell (DC) function. Although the exact mechanism behind flavonoid inhibitory effects remains unclear, administration of flavonoids seems to be a promising dietary treatment for allergic asthma and food allergy.

Introduction

Allergic disease has become increasingly prominent in the last decade in both developed and developing countries. Up to 4 and 8% of children and adults, respectively, suffer from food allergy in the United States and these numbers appear to be increasing[1,2]. While mostly allergic diseases, such as allergic rhinitis, are not life-threatening, unfortunately every year unavoidable deaths, caused by anaphylaxis and asthmatic attacks, occur[3]. Allergic disease is caused by the inaptness of the immune system, in which it incorrectly triggers an inflammatory response to particular antigens, allergens. Some of the most frequently identified allergens around the world include plant pollen, food, such as nuts, and animal dander[4]. In the early 1900s, Charles Richet discovered that repeated exposure to an antigen may cause sensitization. He discovered this when, he tried to immunize dogs with a jellyfish toxin and some dogs exerted an almost immediate and fatal response to the toxin at repeated exposure[4].

The importance of genetic factors in allergic disease have been explored in genome-wide association studies (GWAS) and several genes, such as T-helper 2 (Th2) cytokine genes,

were found to be important in allergic disease[5]. On the other hand, environmental factors, such as diet, are thought to possibly play an even more significant role in allergic diseases than genetic factors[5]. Environmental factors are a more probably cause for the recent rise in allergic disease, since the likelihood of a change in genetic makeup within one or two generations is very low.

Allergic sensitization occurs at major epithelial barriers, such as the lung and gut, because this is where the body is exposed to the allergen for the first time. Thus, the function of these barriers is of great importance for a proper immune response and their dysfunction is a primary cause of allergic sensitization. Once the body recognizes the allergen, there are two main phases to allergic sensitization; the primary response and the memory phase. In the primary response, the epithelial cells are activated by the allergen and pro-inflammatory mediators are released. This causes the activation and maturation of dendritic cells (DCs), which are trafficked to tissue draining lymph nodes, where the naïve T cells are activated. These cells differentiate into T helper-2 (Th2) effector cells, which are released into circulation. While most of these cells die, a fraction of them differentiate into memory T cells (Tm) a few weeks after the initial inflammation; part of the memory phase. Allergen-specific immunoglobulin-E (IgE) is produced by B cells due to Th2 cytokine release. Upon re-encounter with the allergen, IgE cross-linking on effector cells, such as mast cells, causes the various symptoms of allergy[6]. In the initial process of allergic sensitization and in the later response, a group of chemotactic cytokines, known as chemokines, are important mediators of the inflammation[7–9].

Chemokines

Over 40 different types of chemokines and chemokine receptors play a role in allergic disease, specifically in recruiting and trafficking of inflammatory cells[7–9]. Targeting chemokine receptors with small molecule antagonists, monoclonal antibodies and peptide-derived antagonists started off as an attractive avenue for treating allergic inflammation. This review provides an overview of the antagonists that have been developed thus far and their efficacy in animal models. A few of these antagonists have gone to clinical trials. Even so, the need for a novel therapeutic approach has risen. A possible solution may be to use dietary intervention, for example with flavonoids, which generates a multi-target approach targeting several chemokines and their receptors instead of just one.

Flavonoids

Biologically active polyphenolic plant secondary metabolites, flavonoids, have been studied for their anti-inflammatory, anti-oxidant and immune-modulating characteristics. The general backbone structure of flavonoids is made of two aromatic rings joined by three carbons making up the oxygenated heterocycle (depicted in Figure 1). Six groups of flavonoids have been identified (listed in Table 1). Flavonoids are highly abundant in many of our food sources, including tea, fruits, nuts, vegetables, wine and coffee[10]. Studies using several of these flavonoids have produced promising results and thus, have led to a rise in interest of possible dietary treatments of several burdening diseases, such as allergic disease. From the several classes of flavonoids, flavonols and isoflavones have been identified as the most effective in inhibiting inflammatory responses[11].

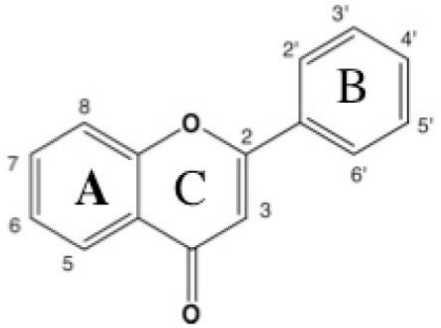

Figure 1 - Backbone structure of flavonoids[10]

Table 1 – Classes of flavonoids[10]

Flavonoid class	Members of the class
Flavones	Luteolin, apigenin, baicalein, acacetin
Flavonols	Fisetin, kaempferol, quercetin, galangin, myricetin
Flavanones	Hesperetin, naringenin, eriodictyol, sakuranetin
Isoflavones	Daidzein, genistein
Flavanols	Catechins, proanthocyanidins
Anthocyanidins	Cyanidin, pelargonidin

In this review, we focus on chemokines and chemokine receptors in allergic asthma and food allergy, and the potential of using flavonoids in the prevention of allergic sensitization and/or inflammation through inhibition of chemokine function. To do so, we first elaborate on the mechanisms of allergic disease and chemokines, and then go into detail on the different studies investigating inhibitory effects of flavonoids in allergic disease.

Mechanisms of allergic disease: focus on asthma and food allergy

Allergic disease, or Type I hypersensitivity, is caused by an IgE-mediated response, in which mucosal mast cells are activated. This results in the elicitation of allergic symptoms and consequently in an increase in activated $CD4^+$ Th2 lymphocytes. Elicitation of allergic inflammation is a complex process, in which various cellular components, such as DCs, mast cells and T cells, inflammatory mediators, such as cytokines and chemokines, and structural cells, such as epithelial cells, play a part[5].

Epithelial barriers of the lung and gut are exposed to environmental factors and thus, are the first line of defense against pathogens. This is also where allergic sensitization is developed (depicted in Figure 2). Allergic sensitization occurs due to the malfunction of the epithelial barriers and therefore, the allergen is able to activate the epithelial cells[6]. The epithelial cells express various pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs). The transcription factor, nuclear factor-κB (NF-κB), is activated by the majority of these signaling pathways and is responsible for expressing various cytokine and chemokine genes. It has been shown that activation of epithelial NF-κB is a crucial aspect of airway allergen sensitization[12]. The epithelial cells express inflammatory mediators, such as granulocyte-macrophage colony stimulating factor (GM-CSF), Interleukin-25 (IL-25), IL-33 and thymic stromal lymphopoeitin (TSLP). These mediators are important for the activation of DCs and naïve T cells. The allergen can also directly activate DCs if it passes the epithelium as an intact protein. DCs are an important part of the immune response to allergens, as they present the allergen to the Th2 cells inducing the primary Th2 response[2].

The Th2 cells are recruited to the site of allergic inflammation and activated, after which they release pro-inflammatory cytokines IL-4 and IL-13. The regulation of Th2 cell recruitment and activation is controlled by the secretion of chemokines CCL17 and CCL22 by DCs[5]. While Th2 cells are the main T cells driving allergic inflammation, several other ones have been implicated, such as Th1, $CD8^+$ and natural killer (NK) T cells. Interestingly, regulatory T cells (Tregs) are thought to suppress allergic inflammation, by direct contact with DCs or by secreting IL-10 or transforming growth factor–beta (TGF-β). People suffering from allergic diseases possibly have impaired Treg function. The secretion of cytokines by the Th2 cells causes the activation of IgE production by B cells. However, the function of the various B cells is still unclear[5].

In the innate immune response to an allergen, the activation of mucosal mast cells plays an important role. The interaction of IgE with an allergen causes mast cell degranulation and subsequent release of mediators, such as histamine, tumor necrosis factor alpha (TNFα) and

chemokine CCL1, which recruit Th2 cells. This process causes bronchoconstriction, vasodilatation and exudation of plasma and consequently, clinical symptoms of asthma[5]. In food allergy the symptoms can range from respiratory to gastrointestinal to dermatological symptoms[13]. To maintain the mast cells at the mucosal site of inflammation, cytokines are released by epithelial cells. However, the function of mast cells in later phases of allergic disease is still unclear[5].

In the late phase response of allergic airway inflammation, the allergen causes epithelial cells and macrophages to release various chemokines, including CCL20 and CXCL10, which in return recruit T cells to the sites of inflammation. Also, DCs release chemokines CCL17 and CCL22 due to activation by IL-4 and IL-13 and thus, recruit Teff cells. Eosinophils, activated by chemokines CCL24 and CCL26, are also important in allergic inflammation and are regulated by Th2 cells releasing IL-5. The eosinophils release inflammatory mediators, sometimes function as APCs and produce Th2 and Th1 cytokines. Eosinophils are not as crucial as mast cells in the immediate allergic response, but appear to be more important for the late phase allergic inflammation. On the other side, the role of neutrophils in allergic inflammation also remains unknown[5]. In food allergy, the role of specific chemokines in allergic sensitization and the effector response in the intestinal mucosa is remains relatively undefined. Only CCR9 and its ligand CCL25 are known to be specific for the gut. Through interaction with CCL25, gut Teff cells expressing CCR9 can enter the small intestine[6].

In allergic asthma and food allergy, the bronchial and intestinal epithelial barriers and DC activation are important aspects of the mechanism of action of the diseases. Therefore, we focus on the processes occurring at these sites and the chemokine involvement therein.

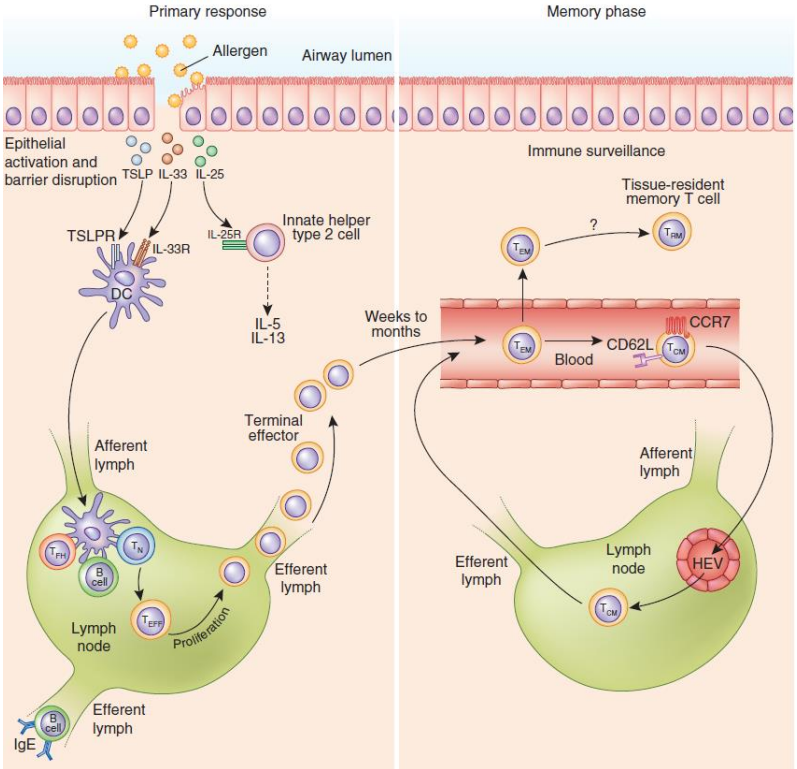

Figure 2 – Primary response to an allergen. The allergen activated epithelial cells and pro-inflammatory mediators TSLP, IL-33 and IL-25 are released. Naïve DCs are activated and trafficked to the tissue draining lymph nodes, where T cells differentiate into effector T (Teff) cells. The Teff cells are released into the circulation. In the memory phase, a fraction of the Teff cells differentiate into memory T (Tm) cells a few weeks after the initial response[6].

Allergic asthma

The bronchial epithelial barrier is an important factor in the development of allergic asthma. It was previously thought to be of little importance in pulmonary immunity, but actually it is a crucial aspect of both the innate and adaptive immune systems[14]. The barrier consists of a single layer of epithelial cells covered by a periciliary layer, that protects the microvilli, and a mucous layer, that traps unwanted particles or pathogens. Studies have shown that the epithelial barrier in asthmatic patients is more prone to injury and is unable to repair itself. Furthermore, the tight-junctions are gravely impaired[15]. E-cadherin is an important aspect of the adherens junctions and is shown to be reduced in asthmatic patients. However, whether reduced E-cadherin levels is a cause or effect of allergic asthma still remains unknown[12]. Due to these factors, allergens may be able to penetrate the epithelial barrier more easily and thus, cause sensitization and inflammation. DCs are important messengers between the innate and adaptive immunity. In sensitization, DCs act as APCs and induce activation of naïve T cells to Th2 effector cells. In late phase allergic inflammation DCs and macrophages are involved in trafficking T cells[6].

In allergic asthma, Th2 cells and mast cells are activated and in return, pro-inflammatory mediators are released, causing symptoms such as, vascular leakage, bronchial smooth muscle contraction, airway hyper-responsiveness and airway remodeling[8]. Increased levels of mast cells, eosinophils and Th2 cells are observed in lung biopsies of asthmatic patients. Due to the Th2 biased response in many asthmatic patients, therapeutic methods targeting the Th2 cytokine production have been developed. However, they are only effective in a subset of patients, since in some cases other types of T cells are primarily involved. On the other hand, in close to all cases of asthma, airway remodeling takes place due to changes in the airway barrier structure. Two causes of these changes are hyperplasia and hypertrophy, which increase the airway smooth muscle mass due to the growth factors released by airway inflammatory and epithelial cells[5]. Other factors include, elevated levels of mucous producing Goblet cells, loss of airway integrity and increased barrier thickness due to accumulation of extracellular matrix components beneath the epithelial membrane[12].

Food allergy

Food allergy is developed due to the improper function of the epithelial barrier of the gut. The function of the gastrointestinal mucosal barrier is to prevent the invasion of pathogens into the body and it is divided into physiological and immunological constituents[1]. Epithelial cells, connected by tight junctions, form the single layer of the physiological barrier. A surrounding thick mucosal layer traps foreign particles, bacteria and viruses[1]. The immunological barrier is divided into innate and adaptor cells and factors. An oral tolerance, mainly accomplished by the epithelial cells, DCs and Treg cells, needs to be developed since the mucosal immune system is exposed to large amounts of antigens on a regular basis[1]. However, sometimes problems with the gastrointestinal mucosal barrier cause the opposite to happen. The early onset of food allergies in infants may be due to the immaturity of the different barrier components, but these allergies normally dissipate over time[13]. However, recent studies have shown that changes to the physiological barrier, such as pH, can lead to IgE sensitization and thus, food allergy. Furthermore, increased intestinal permeability exposes the body to intact proteins, leading to more severe reactions[1,13].

Food allergen sensitization seems to be caused by the uptake of allergens by the Peyer's patches or by transcytosis via enterocytes. It is further developed, when the allergens are transported across the epithelial barrier and thus, are exposed to various cellular components as intact proteins[16]. The allergens can directly activate DCs and induce Th2 skewing or indirect activation of DCs can occur through DC interaction with epithelial cells. This causes the release

of various pro-inflammatory mediators and recruitment of Th2 cells[2]. While in this review, IgE-mediated food allergy was mainly discussed, non-IgE mediated food allergy also exists. The understanding behind the mechanism of non-IgE mediated food allergy is still fairly limited. A few studies suggest that an increase tumor necrosis factor-alpha (TNF-α) and a decrease TGF-β1 responses are indicators of this type of food allergy[1].

T cell homing to epithelial barriers

Homing of T cells to epithelial barriers is another important concept in allergic disease. At the onset of inflammation, adhesion molecule ligands, such as chemokines, are up-regulated and direct T cells, with the corresponding receptor, to the site of inflammation. Consequently, a tissue-specific "imprinting" process takes place to increase the T cell specificity to an allergy prone organ, such as the small intestine. This way tissue-resident memory T (Trm) cells are formed. Trm cells have been shown to remain at uninflamed peripheral tissue sites, such as the lungs and gut, for extended periods of time by several studies (reviewed by Islam SA and Luster AD)[6]. The significance of this in allergic disease is that the chances of a T cell coming into contact with an activated DC presenting an allergen are increased and this causes the generation of more Tm cells and long-lived Trm cells specific for the allergen. Thus, even more of these T cells are generated and the likelihood of encounter with allergen activated DCs becomes even higher[6]. In summary, the homing of T cells to specific tissue sites causes more efficient immune reactions, which is good for the defense against unwanted pathogens, but the opposite for allergic disease, as the reactions will be faster and more severe.

Chemokines and chemokine receptors: their role in allergic disease

Chemokines and chemokine receptors are major players in allergic disease. Chemokines are a group of small proteins of 8 to 10 kDa with a high affinity to heparin and bind specifically to G-protein coupled receptors (GPCRs)[5,9].

Chemokines are classified into several groups according to the four cysteine residues in their polypeptide chain. Four groups of chemokines have been identified; the CC or beta chemokines (two adjacent cysteines in the amino terminal), the CXC or alpha chemokines (an amino acid separates the first two cysteines), the C or gamma chemokines (lack two cysteines), and the CX3C chemokines (three amino acids separate the first two cysteines). Chemokine receptors are also classified into four groups. The shared receptors are receptors that bind several different chemokines of the CC or CXC families, the specific receptors are ones that bind only one chemokine, the promiscuous receptors are ones that bind chemokines of either CC or CXC families, and the virus-codified receptors are ones that are codified in virus genomes (listed in Table 2)[7–9]. The table also lists all chemokine receptors and the chemokine ligands that bind these receptors. Chemokine receptors have very dynamic surfaces, a feature which is key to their ability to recruit leukocytes to the site of inflammation and also, to traffic DCs and T cells to secondary lymphoid organs[9]. This shows how chemokines are crucial in both innate and adaptive immunity. The CC and CXC chemokines make up the majority of the chemokines known today[9]. Hence, mainly the role of the chemokines in these two families and their receptors have been studied in allergic disease.

Function of chemokines in allergic disease

Several chemokines and their receptors play roles in allergic disease. As mentioned earlier, in the early phase of allergic inflammation, the allergen interaction with IgE causes mast cells to release CCL1, which recruits Th2 cells by binding its receptor CCR8. CCR8 is expressed on approximately 70% of Th2 cells and in asthmatic patients a higher prevalence of these cells is observed. Furthermore, the importance of the CCL1/CCR8 axis was shown *in vivo*. Mast cell deficient mice

showed decreased levels of CCL1 and reduced asthmatic symptoms and inhibiting CCR8 function also suppressed the symptoms[17].

Overall, the CCL1/CCR8 axis is the most crucial chemokine system in the early phase of allergic inflammation. However, CCR8 is probably involved in Th2 recruitment to the site of inflammation also in the late effector phase since it binds other ligands as well (CCL4, CCL16 and CCL17), but as of yet there is no concrete data to support this claim[17]. Other chemokines and their receptors play vital roles in the late T-cell mediated effector phase. In the late phase, macrophages and epithelial cells release chemokines CCL20 and CXCL10 upon TLR activation by an allergen. Various T lymphocytes are recruited by these chemokines via their receptors CCR6 and CXCR3, respectively. CCR6 is a regulator of lung and gut immunity and is expressed on many different cell types, making it important in both innate and adaptive immunity.

Table 2 – Chemokine families: the ligands and receptors belonging to each family and the cells that express the receptors[7–9].

Family	Ligand	Receptor	Cell Expressing Receptor
CC (beta)	CCL3 (MIP-1α), CCL5 (RANTES), CCL7, CCL8, CCL9/10, CCL13, CCL14, CCL15, CCL16, CCL17, CCL23	CCR1	Ba, DC, Eo, Mo, T, PMN, NK
	CCL2 (MCP-1), CCL7 (MCP-2), CCL8 (MCP-3), CCL13 (MCP-4)	CCR2	Mo, DC, T, Ba
	CCL5 (RANTES), CCL7, CCL11 (eotaxin-1), CCL13, CCL15, CCL16, CCL24 (eotaxin-2), CCL26 (eotaxin-3)	CCR3	Eo, T, Ba, MC
	CCL17 (TARC), CCL22 (MDC)	CCR4	DC, T, Ba, NK
	CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL8	CCR5	Mo, DC, T
	CCL20 (LARC/MIP-3κ)	CCR6	DC, T
	CCL19 (ELC), CCL21 (SLC)	CCR7	DC, T, B, NK
	CCL1 (I-309), CCL4 (MIP-1β), CCL16, CCL17	CCR8	Mo, T, NK
	CCL25 (TECK)	CCR9	T
	CCL27 (CTACK), CCL28 (MEC)	CCR10	T
	CCL912	Unknown	
	CCL18 (PARC)	Unknown	
CXC (alpha)	CXCL6 (GCP-2), CXCL7 (NAP-2), CXCL8 (IL-8)	CXCR1	N, Mo
	CXCL1 (GRO-α), CXCL2 (GRO-β), CXCL3 (GRO-γ), CXCL4 (PF-4), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (NAP-2), CXCL8 (IL-8)	CXCR2	N, Mo
	CXCL9 (MiG), CXCL10 (IP-10), CXCL11 (iTAC)	CXCR3	T, B
	CXCL12 (SDF-1)	CXCR4	T, B, DC, Mo
	CXCL13 (BLC)	CXCR5	T, B
	CXCL14 (BRAK), CXCL15 (lungkine)	Unknown	
	CXCL16	CXCR6	T
	CXCL17 (DMC)	CXCR7	None
C (gamma)	CL1, CL2	CR1	NK, T
CX3C	CX3CL1 (Fractalkine)	CX3CR1	Mo, NK, T, DC

Ba basophil, *DC* dendritic cell, *Eo* eosinophil, *Mo* monocyte, *T* T cell, *PMN* polymorphonuclear cell, *NK* natural killer cell, *MC* mast cell, *B* B cell, *N* neutrophil.

CCR6 functions include, recruiting naïve and mature DCs and trafficking of Th cells to the mucosal lymphoid tissue. Mouse models of allergic airway inflammation have shown that CCR6 is crucial for DC recruitment to the lungs and that exposure to an allergen significantly elevates CCL20 levels[18]. CXCR3, expressed by T cells, binds two other ligands, besides CXCL10, CXCL9 and CXCL11. The interaction with CXCL10 is shown to cause the recruitment of T cells into the airway[6]. CXCR3 is associated more with the Th1 response than the Th2 response, because the ligand production is triggered by interferon-gamma (IFNγ). Interestingly, the ligands are antagonists of CCR3, which is a Th2 response mediator[8].

CXCL8 and its receptors CXCR1 and CXCR2 have also been implicated in late phase allergic inflammation as a chemoattractant for eosinophils and neutrophils[9,19]. CCR5 recruits and activates eosinophils through interaction with its ligand CCL5, which is another important chemokine/receptor axis in late phase allergic inflammation[20]. Also in the late effector phase, CCL17 and CCL22 are released by DCs due to the release of IL-4 and IL-13 by allergen activated Trm cells and this causes the recruitment of Th2 effector cells via CCR4[6]. Mikhak *et al.* determined the role of CCR4 in trafficking of antigen-specific Th2 cells in a CCR4 deficient allergic pulmonary inflammation mouse model. The results showed that in the deficient model Th2 cells were significantly less efficient at trafficking to the sites of inflammation. Thus, this suggests that CCR4 is a crucial part of antigen-specific Th2 cell trafficking to the lungs[21].

CCR7 and the corresponding ligands, CCL19 and CCL21, take part in DC, T cell and B cell trafficking, shown with CCR7$^{-/-}$ mice. Furthermore, recently Afshar *et al.* showed the importance of CCR7 and CCR4 in allergic pulmonary inflammation. The results indicate that Tregs need CCR7 in order to suppress sensitization, while Tregs need CCR4 for the suppression of the recall response. Thus, it was stipulated that because inhibiting Teff cells also causes the inhibition of the Treg cells, recent therapeutics targeting these receptors may not have been effective. Therefore, therapeutics targeting specifically the receptors on the Teff cells should be taken into consideration and further research needs to be done on this subject[22].

The ligands CCL11, CCL24 and CCL26 are released by epithelial cells and macrophages upon allergen activation in the late effector phase. The ligands recruit eosinophils through the corresponding receptor CCR3, expressed on eosinophils. According to the findings of Heiman *et al.*, CCL11 is produced in the initial 12 hours of stimulation with an allergen while CCL24 and CCL26 are produced after 12 to 48 hours[23]. This may suggest that CCL11 is important in the initial requirement of eosinophils whereas CCL24 and CCL26 are needed for the ongoing recruitment. Fulkerson *et al.* showed with several different mouse models that the activation of eosinophils via CCR3 is an important aspect of allergic inflammation. The results suggest that eosinophils can regulate Th2 cytokine release and that suppression of either CCR3 or depletion of eosinophils could be used as a therapeutic means[24].

The CCL25/CCR9 axis has been identified as gut-specific, in which the Teff cells are imprinted with CCR9. Upon inflammatory activation, the epithelial cells produce CCL25, attracting the CCR9 imprinted T cells to the site of inflammation[6,25]. While this chemokine interaction could be important for therapeutics measures in food allergy, at this point only a limited number of studies have been conducted and therefore, this needs to be further investigated. The study by Tubo *et al.* provided evidence for the possibility of tissue-specific therapy by targeting CCR9. In the mouse model for gut and skin inflammation, an antagonist for CCR9 caused for CD8+ T cells to not accumulate at the

small intestine epithelial barrier, but the antagonist had no effect on the T cells at the site of skin inflammation[26].

Several other chemokine receptors have been implicated in allergic inflammation, such as CCR2 and CXCR4. CCR2 binds ligands of the macrophage chemotactic protein chemokine family, including CCL2. CCL2 is indicated in mast cell recruitment and activation (reviewed by Velazquez and Teran)[8]. The CCR2/CCL2 axis has been shown to be important in mouse models for allergic asthma by several studies (reviewed by Mellado *et al.*)[27]. Levels of CXCR4, binding only the CCL12 ligand, are increased on Th2 and eosinophil cell surfaces during airway inflammation in asthmatic patients. CXCR4 probably functions as a recruiter of leukocytes to the site of inflammation[8,9].

Interestingly, while most chemokines and chemokine receptors enhance allergic inflammation, Bellinghausen *et al.* recently published results of a study indicating that CCL18 production by tolerogenic DCs inhibits allergic airway reactivity. This was tested in a humanized mouse model for allergy as well as in *in vitro* studies. The results showed that CCL18 recruited Treg cells more effectively than Th2 cells. Thus, CCL18 is another possible therapeutic target for allergic diseases. However, the receptor of this chemokine is still to be discovered[28].

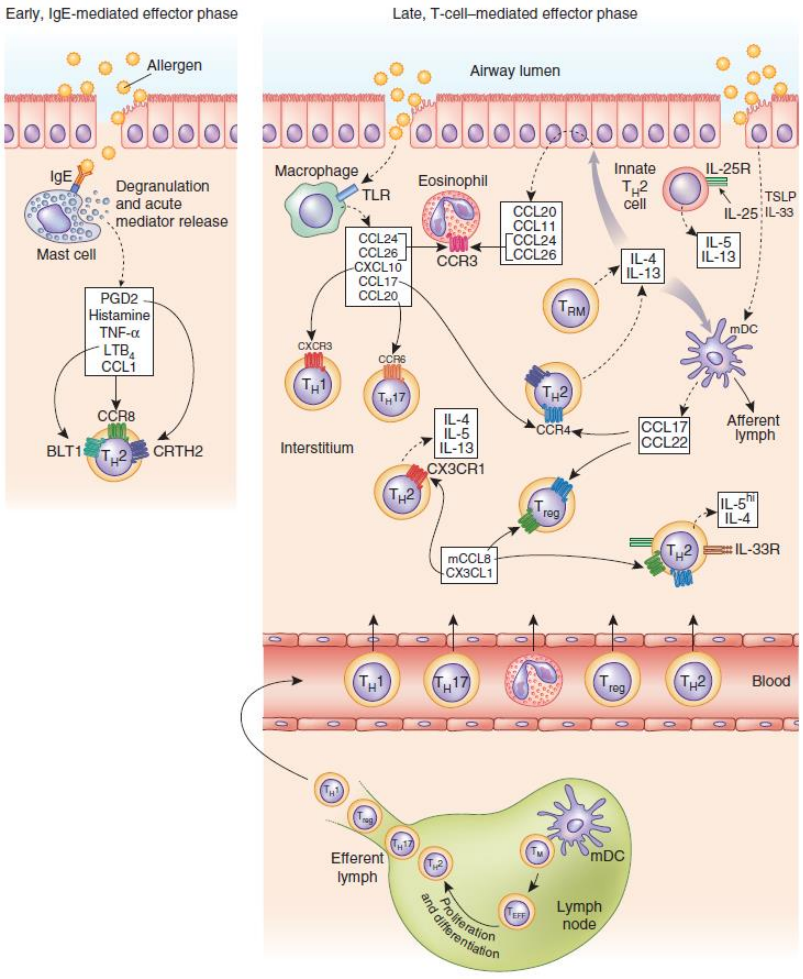

Figure 3 – In the early, immediate IgE-mediated response, the IgE interaction with the allergen causes the degranulation of mucosal mast cells and the release of mediators, PGD2, Histamine, TNF-α, LTB_4 and CCL1. These mediators recruit T-helper 2 (Th2) cells via their respective receptors. In the late, T-cell mediated effector phase, the activation of toll-like receptor (TLR) causes the release of chemokines CXCL10 and CCL20, which recruit T cells via their receptors CXCR3 and CCR6. The myeloid dendritic cells (mDCs) release CCL17 and CCL20 upon activation, which recruit more Teff cells via their receptor CCR4.[6]

Chemokine antagonists developed thus far for allergic disease

Several studies have been conducted into the inhibition of chemokines or their receptors with small molecules, monoclonal antibodies or peptide-derived antagonists. Several of these antagonists have proven efficacious in animal models of allergic asthma[8,27,29,30]. Currently, there are no studies available for chemokine antagonists in the prevention or treatment of food allergy. Not all of the existing antagonists are discussed below, only some of the more recent ones.

Komai *et al.* studied the effects of a CCR3 antagonist, Ki19003, in an ovalbumin sensitized BALB/c mouse model for allergic asthma. The results showed that inhibiting CCR3 reduces eosinophil levels in the airway and subepithelial and peribronchial fibrosis *in vivo*. However, the antagonist had no effect on CCR2 and CCR7 induced chemotaxis. Furthermore, while there was a significant decrease in airway responsiveness using the antagonist, it was still not as effective as prednisolone[30]. Another study by Tian *et al.* found that a peptide, CKLF1-C19, successfully inhibits chemotaxis mediated by CCR3 and CCR4 in a humanized mouse model for asthma. The peptide decreased airway hyperresponsiveness, lung inflammation and airway eosinophilia[29]. A CCR3 antagonist developed by Shering Plough entered clinical trials but caused some unwanted inhibitory effects and thus, the trials were arrested. Another CCR3 antagonist developed by GlaxoSmithKline entered clinical trials after promising *in vivo* results, yet showed inefficacious in a phase III trial[9].

As previously discussed, CCR4 is important in allergic inflammation due to its recruitment of antigen-specific Th2 cells to the site of inflammation through its ligands CCL17 and CCL22. Sato *et al.* investigated the effects of a CCR4 antagonist, K327, on airway inflammation in an ovalbumin sensitized BALB/c mouse model. The antagonist successfully suppressed the recruitment of Th2 cells by CCR4 in a dose-dependent manner. Furthermore, decreased levels of Th2 pro-inflammatory cytokines and airway eosinophilia were observed *in vivo* after the treatment with K327[31]. Thus, CCR4 is another potentially effective therapeutic target in allergic disease.

In the early phase of inflammation, CCR8 and CCL1 have been implicated, as explained before. Previous studies on the CCL1/CCR8 axis have resulted in contradictory data (reviewed by Velazquez and Teran)[8]. Thus, Wang et al. tested a CCR8 inhibitor, ML604086, in a non-human primate model, cynomolgus monkey, for allergic asthma. While the antagonist successfully inhibited CCL1 binding to CCR8 on T cells, it had no effect on airway eosinophilia, pro-inflammatory cytokine production or airway resistance and compliance[32]. Therefore, while CCR8 has been shown to be of importance in the allergic inflammation pathway, inhibiting it seems to have no beneficial therapeutic effect.

CXCR3 and CCR5 have also been targeted by small molecular antagonists. Suzaki *et al.* studied the effects of TK-779 on airway inflammation in an ovalbumin sensitized mouse model. Upon treatment with the antagonist, decreased levels of CXCR3 and CCR5 were found on circulating T cells and further, airway responsiveness and inflammation were suppressed[33].

The CXCL12/CXCR4 axis has caused some debate over its role in allergic inflammation. However, antagonizing either the receptors or ligand decreases asthmatic symptoms, such as airway hyperreactivity, in mice[8,9]. The issue with inhibiting CXCL12/CXCR4 function is that the chemokine and its receptor are also involved in other important processes, besides inflammation, such as normal tissue patterning, cardiac and neuronal development, angiogenesis and tumorigenesis.[8,9,34]. This in mind, Daubeuf *et al.* developed a soft drug, neutraligand, which blocks the interaction between CXCL12 and CXCR4. The drug was

delivered intranasally in a mouse model of asthma and in this way selectively reduced airway eosinophilia without inhibiting the target in other parts of the mice[34]. Another antagonist, AMD3100, for CXCR4 has been developed by Genzyme. However, this molecule inhibits CXCR4 function systematically and therefore, increases the probability of undesirable side effects[34].

While several antagonists seemed promising in *in vitro* studies and some even in *in vivo* models, very few have continued onto trials in humans[35]. Many CCR3 antagonists have been developed, while there are other receptor/ligand axis also very important in allergic inflammation and should be considered for therapeutic purposes. Furthermore, none of these studies focused on food allergy, but particularly on allergic asthma. Other therapeutic means to inhibit chemokine function, such as dietary intervention, should be investigated. In addition, most of the antagonist developed so far target only one chemokine/receptor axis and with dietary intervention the possibility of a multi-target approach arises.

Modulation of chemokines through dietary interventions

Recently, interest in using dietary intervention as a therapeutic mean has grown immensely. Flavonoids have drawn particular attention in relation to allergic disease due to their anti-inflammatory effects. NF-κB is an important aspect of allergic inflammation because upon allergen activation, epithelial cells activate the NF-κB pathway, which is known to regulate expression of various pro-inflammatory mediators, including chemokines. Therefore, this pathway is important for proper chemokine function. Other pathways such as the mitogen-activated protein kinase (MAPK) pathways have also been implicated in allergic inflammation, but activation of the NF- κB pathway has been shown to be sufficient to cause allergic inflammation (reviewed by Lambrecht and Hammad)[12]. The NF-κB pathway is targeted by flavonoids, according to several studies (reviewed by Tanaka and Takahashi)[10]. Here, we discuss studies in which several flavonoids have been investigated for their inhibitory effects in *in vitro* and *in vivo* allergic disease models.

Apigenin was investigated for its effect on chemokine production by LPS-induced human THP-1 monocyte cells by Huang *et al.*. Interestingly, while previous studies have showed that the chemokine inhibition occurs through the NF-κB pathway, in this study the results suggest that the inhibition is rather through MAPK pathways[36]. Another study by Huang *et al.* indicates that kaempferol is an inhibitor of chemokine expression in THP-1 cells. The results show that kaempferol suppresses the production of CXCL10, CCL11, CXCL8 and CCL1, all of which are crucial in allergic inflammation. The results of this study also suggest that the inhibitory effects of kaempferol are through MAPK pathways in THP-1 cells[37].

According to a study by Hamalainen *et al.*, kaempferol, quercetin, genistein and daidzein inhibit the activation of NF-κB in murine macrophages exposed to LPS. The effectiveness of the flavonoids was attributed to particular structural components and their anti-oxidant effects. A C-2,3 double bond is found in the most effective inhibitors, a bulky group substituent decreased the inhibitory effects (shown with quercetin), and in all inhibitors there were 7 and 4' hydroxyl groups[11].

Another flavonoid, galangin, has also been shown to exhibit inhibitory effects on mast cell mediated allergic inflammation *in vitro* and *in vivo*. Galangin suppressed the expression of CXCL8, along with other pro-inflammatory cytokines, and attenuated cutaneous anaphylaxis with better efficacy than cromolyn, anti-allergic drug on the market. It was speculated that the inhibitory effects of galangin were due to regulation of the NF-κB, MAPK and caspase-1 pathways[38].

Lee *et al.* investigated the effects of two flavonols, quercetin and kaempferol on IgE-mediated allergic inflammation in two cell lines, RBL-2H3 (rat basophilic leukemia) and Caco-2 (human colon adenocarcinoma), often used to study the intestinal epithelial barrier. The barrier is an important part in allergic sensitization and inflammation and thus, of interest in studying food allergy. RBL-2H3 cells express the FcεRI receptors, which have a high affinity for IgE, release chemical mediators, and they are similar to mucosal mast cells. The Caco-2 cell line, on the other hand, is employed because of its high polarized brush border and tight junction. The *in vitro* results suggest that quercetin has an inhibitory effect on the release of CXCL8 and CCL20. Furthermore, the inhibitory effects of quercetin and kaempferol on the expression of low affinity IgE receptor CD23 caused significantly less chemokine release upon IgE-allergen crosslinking and could possibly decrease the extent of allergic inflammation[19].

Polyphenol abundant apple extract contains quercetin, amongst other flavonoids, and has been studied for its anti-inflammatory effects in food allergy. In an ovalbumin sensitized BALB/c mouse model for food allergy, a reduction in clinical symptoms and in CCL11 levels were observed when the already sensitized mice were administered the apple extract for a week before oral challenge. However, these effects were not seen when the apple extract was administered during sensitization[39]. Therefore, the apple extract appears to prevent allergic inflammation rather than sensitization.

Acacetin is another plant flavonoid that has been investigated for its anti-inflammatory effects in an ovalbumin sensitized BALB/c mouse model for asthma. The flavonoid reduced airway hyperresponsiveness, goblet cell hyperplasia and eosinophil assembly and decreased levels of CCL11 were found in the bronchoalveolar fluid. Furthermore, in *in vitro* studies using human bronchial epithelial (BEAS-2B) cells, acacetin reduced chemokine CCL11 and CXCL8 levels, along with other cytokines[40].

Nanua *et al.* demonstrated *in vitro* and *in vivo* the inhibitory effects of quercetin on CXCL8 and CCL2 production by airway epithelial cells. The *in vitro* results suggest that this is due to blocking of the NK-κB pathway and the *in vivo* results indicate a therapeutic effect of quercetin on airway inflammation. The results suggest that both transcriptional and post-transcriptional pathway chemokine expression are suppressed by quercetin[41]. Kempuraj *et al.* also showed that quercetin and kaempferol suppressed CXCL8 release by approximately 90%, in human mast cells[42]. The effects of quercetin were further investigated in a study by Castellani *et al.*, where quercetin inhibited the release of CCL2 in a dose-dependent manner, in a human mast cell line stimulated by physiological and non-physiological activators[43].

The results of Gong *et al.* showed *in vitro* that kaempferol suppresses the expression of several pro-inflammatory mediators, such as CCL2 and CCL11, and *in vivo* that kaempferol disrupts NF-κB signaling and thus, reduces airway inflammation. It is possible that the suppression of the NF-κB down-regulated the production of the mediators and consequently, lower levels of them were found[44].

Another flavonol potentially inhibiting NF-κB function is fisetin. In a study by Wu *et al.* fisetin is shown to suppress lung inflammation, goblet cell hyperplaisia and airway hyperresponsiveness in an ovalbumin sensitized mouse model. The inhibitory effects are attributed to the decreased Th2-associated regulators and bronchial epithelial TSLP and CCL11 and suppressed NF-κB activation in lung tissue and bronchial epithelial cells[45]. These results were supported by another study by Goh *et al.* testing a female BALB/c mouse model sensitized to ovalbumin and then challenged. They suggest that the symptoms of asthma, mucus hypersecretion and airway hyperresponsiveness, were

significantly reduced due to suppression of the NK-κB signaling pathway[46].

Bao *et al.* investigated the anti-inflammatory effects of soy isoflavone. The study showed that soy isoflavones attenuate airway inflammation in an ovalbumin sensitized mouse model for allergic asthma. Airway hyperresponsiveness along with eosinophil infiltration were reduced by soy isoflavone treatment. Furthermore, decreased levels of CCL11, amongst other pro-inflammatory mediators, were found[47].

Toledo *et al.* investigated the inhibitory effects of another flavonoid, sakuranetin, on allergic asthma. Ovalbumin sensitized male BALB/c mice were administered sakuranetin for six days before determination of airway hyperresponsiveness, inflammation and remodeling. The results indicate that sakuranetin reduces these symptoms and decreases expression of CCL5 and CCL11 (associated with eosinophil infiltration) as well as other cytokines. These effects may have been due to the suppression of NF-κB activation[48].

In another study, silibinin was found to exhibit inhibitory effects *in vivo*. Ovalbumin sensitized mice, treated with silibinin prior to each allergen challenge, showed reduced airway hyperresponsiveness, airway inflammatory cell recruitment and pro-inflammatory mediator production. It was stipulated that this is probably (partially) due to suppression of the NF-κB pathway[49].

In order to understand more in depth the function of flavonoids as a therapeutic mean for allergic disease, the effect of flavonoids on DC function is discussed, because DC function is directly related to chemokine function. DCs have been proven to produce chemokines CCL17 and CCL22 upon activation by TSLP and immature DCs express CCR6 which interacts with its ligand CCL20 upon allergen activation of epithelial cells[2,6,50]. Thus, DC function is closely related to chemokine function.

Mechanism of flavonoids: DC function

Extensive research has recently been conducted into the mechanisms by which flavonoids suppress allergic inflammation. Thus far, several groups have shown this mechanism to be through the regulation of DC function. As previously explained, DCs are crucial for chemokine function and in allergic sensitization and inflammation. Blocking parts of DC function could be promising in the treatment of allergic diseases.

Masilamani *et al.* showed that soybean isoflavones, genistein and daidzein, can inhibit allergic sensitization to peanuts via regulation of DC function. A reduction in CD83 and CD80 levels suggests that the isoflavones suppressed DC maturation and thus, also cytokine secretion from DCs and $CD4^+$ T cells. Furthermore, the isoflavones suppressed the release of pro-inflammatory mediators, IL-6 and CXCL8[51]. Yum *et al.* studied this further and found that daidzein suppresses DC maturation and function by inhibiting CD80, CD86 and MHC class II expression[52]. Similar results were obtained by Masilamani *et al.* in another study in which genistein and daidzein suppressed the expression of CD80, CD83, CD86 and MHC class I proteins *in vitro*. Contrary to the study by Yum *et al.*, no suppression of MHC class II protein expression was observed[53].

Kaempferol was also shown to inhibit DC function in a study by Lin *et al.* The group studied the effects of *Semen cuscutae*, a Chinese traditional medicine, on mouse bone marrow derived DC activation and maturation. They found that kaempferol was the major flavonoid in the *Semen cuscutae* and the inhibitory effects were attributed to it. The production of chemokines CCL2, CCL4 and CCL5 by LPS-induced DCs was significantly decreased in a dose-dependent manner. Furthermore, *in vitro* and *in vivo* allergen-specific T cell activation by the LPS-induced DCs was suppressed by kaempferol[54].

Huang *et al.* showed that quercetin is a potent suppressor of DC activation and function. The suggested mechanism of action is through activation of the aryl hydrocarbon receptor (AhR), an immunoregulator activating Treg cell differentiation. AhR is highly expressed by DCs and since quercetin is a ligand of AhR, this could be the mechanism by which quercetin suppresses DC function. Contrary to other flavonoids, quercetin was also shown to suppress endocytosis of DCs[55].

As explained before, the role of DCs and the NF-κB pathway are important for proper chemokine function and inhibition of DCs and/or the NF-κB pathway also probably impairs chemokine function. Flavonoids have been proven to inhibit the expression of certain chemokines but the data is still very limited. Therefore, further research into the direct effect of flavonoids on chemokine and chemokine receptor function in allergic disease should be conducted. Moreover, the role of the NF-κB and MAPK pathways remains controversial and additional studies should be done to resolve this. Until now, mainly *in vitro* and *in vivo* animal studies into the effects of flavonoids on allergic disease have been conducted.

Clinical trials using flavonoids

While numerous *in vivo* animal studies have been performed investigating flavonoids in the treatment of allergic disease, especially allergic asthma, very few studies have been conducted to investigate the effect of flavonoids on allergic disease in humans. A few studies investigated the effects on allergic rhinitis. In a study by Miyake *et al.*, the effects of high soy and isoflavone ingestion on symptoms of allergic rhinitis was investigated in a group of pregnant Japanese women. The results indicate a possible reduction in allergic rhinitis prevalence due to high intake of soy and isoflavones[56]. In another study, of Kawai *et al.*, a small group of subjects suffering from cedar pollinosis (a form of allergic rhinitis) were given enzymatically modified isoquercitrin. Although the study group was relatively small, the results show a promising effect of flavonoids on allergic disease[57].

Soy genistein has been shown promising in the reduction of allergic asthma symptoms in animals and thus, a relatively recent study investigating the mechanism behind the effect was performed. In a group of subjects with mild to moderate asthma, soy isoflavone supplementation reduced eosinophil LTC_4 synthesis and eosinophilic inflammation[58]. Furthermore, in a post-hoc analysis study of 300 subjects with poorly controlled asthma it was shown that moderate intake of soy genistein correlates with better lung function and asthma control[59]. Clinical trials investigating the effects of Pycnogenol (mixture of bioflavonoids) on asthmatic patients have been performed. The results indicate a positive effect of Pycnogenol on symptoms of asthma, but further studies need to be conducted to confirm the results with larger subject groups (reviewed by Tanaka and Takahashi)[10].

In another study, purple passion fruit peel extract was orally administered to asthmatic patients. Purple passion fruit peel extract was identified to contain a mixture of bioflavonoids and has been used in areas of South America as a folk medicine for treatment of anxiety, bronchitis and asthma. In this study, the efficacy of the purple passion fruit peel extract was tested and found to improve clinical symptoms, such as shortness of breath and wheezing[60].

Overall, there are still only very few clinical studies on the effects of flavonoids on allergic disease. In order to evaluate the practicality of using flavonoids, the bioavailability and feasibility of using flavonoids are discussed below.

Bioavailability and feasibility of using flavonoids

While flavonoids have been shown to have effective anti-inflammatory properties, this is not very helpful unless the

flavonoids have a moderate to high bioavailability. Unfortunately, flavonoids are characterized by low water solubility, poor absorption and rapid metabolism, which result in low oral bioavailability[61,62]. To overcome the obstacle of low bioavailability, different delivery methods have been investigated, including liposomes, various emulsions and cyclodextrin formulations[61].

Liposomal encapsulation of quercetin and fisetin has been shown to increase bioavailability and efficacy in *in vivo* anti-tumor studies[63,64]. Kang *et al.* examined the efficacy of liposomal encapsulated taxifolin glycoside in the treatment of atopic dermatitis in NC/Nga mice. The results suggest that using liposomal delivery significantly increases the therapeutic effects of the flavonoid and is a promising tool to be investigated[65]. Nonetheless, liposomal encapsulation has yet to be extensively investigated as a delivery system in anti-allergic therapy and thus, further research into potential applications should be conducted.

Lipid nanocapsules (LNCs) have also been examined as a delivery system for flavonoids. LNCs are particularly used for low water soluble and pH sensitive drug candidates. Due to the undesirable properties of flavonoids, Barras *et al.* conducted a study investigating the potential of LNCs loaded with quercetin or (−)-epigallocatechin-3-gallate. The stability and solubility of these flavonoids were significantly improved when LNCs were employed[66]. While this study appears promising, additional studies still need to be conducted with flavonoid-loaded LNCs and their effects in the treatment of allergic disease.

In another study, by Regorio *et al.*, the anti-inflammatory effects of quercetin-loaded microemulsions were investigated in an ovalbumin sensitized mouse model for allergic airway inflammation. Using microemulsions increased the bioavailability of quercetin after oral administration and the quercetin-loaded microemulsions inhibited NF-κB activation. The results suggest that the quercetin-loaded microemulsions reduce symptoms of allergic inflammation in mice and that using microemulsions as a delivery system increases the efficacy of quercetin[67].

Isoda *et al.* also showed the advantages of using water/oil emulsions made from Picholine olive oil (rich in flavonoids) as anti-allergic therapy. The results suggest that the water/oil emulsions decreased the release and expression of pro-inflammatory mediators, such as CXCL8, in RBL-2H3 cells sensitization to dinitriphenylated bovine serum albumin[68]. Nanoemulsions are another possible delivery system for flavonoids, shown by Ragelle *et al.* in a study investigating a fisetin-loaded nanoemulsion in a Lewis lung carcinoma mouse model. The results suggested that using nanoemulsions can significantly increase the bioavailability and antitumor activity of flavonoids, particularly of fisetin in this case[69].

Furthermore, cyclodextrin-flavonoid complexes have been studied to find out whether the bioavailability of the components can be increased in this way. Pralhad *et al.* showed that using beta-cyclodextrin and its derivatives can considerably improve the bioavailability of quercetin[70].

In conclusion, there are several ways to increase the bioavailability of flavonoids, by using one of the delivery systems described above. Therefore, even though flavonoids have undesirable characteristics concerning bioavailability, there are ways to solve this problem and with additional research into the possible delivery systems, using flavonoids in the treatment of allergic diseases is feasible.

Conclusions

Allergic asthma and food allergy affect people world-wide and with no effective cure or treatment available today, new avenues need to be explored. Allergic disease erupts from the inaptness of our immune system, where it recognizes foreign proteins as threats and consequently, triggers an

inappropriate immune response. In the worst case scenario this can lead to life-threatening anaphylaxis and even death.

Chemokines are key players in both allergic sensitization and inflammation. Chemokines, proteins of 8 to 10 kDa, and their respective receptors induce trafficking of leukocytes in inflammatory processes by generating various intracellular signals. The signals are released upon chemokine-receptor interaction and cause cells to migrate in a form called chemotaxis[9]. Therefore, the idea of targeting chemokines and/or their receptors brought about the development of various small molecule antagonists, monoclonal antibodies and peptide-derived antagonists[26,30–32,35]. While some studies have promising results, other possibilities, such as dietary intervention, should be investigated. A possible dietary intervention for the treatment of allergic diseases, recently acquiring interest in the scientific community, is the use of flavonoids.

Flavonoids are biologically active polyphenolic plant secondary metabolites found in various food sources, such as teas, fruits, nuts and vegetables. Flavonoids have been studied for their anti-oxidant, anti-inflammatory and immune-modulating characteristics[10]. According to several studies, reviewed here, flavonoids inhibit the release of various pro-inflammatory mediators, including chemokines, and transcription factors, such as NF-κB. While several *in vitro* and *in vivo* animal studies suggest that flavonoids have protective effects against allergic asthma, only a few have explored their effects in food allergy. Furthermore, only a limited number of studies have been conducted into the effects of flavonoids on allergic disease in humans.

In conclusion, using flavonoids in the treatment of allergic asthma and food allergy appears to be a promising and novel therapeutic tool. Flavonoid inhibitory effects are aspecific and thus, they can inhibit physiological and non-physiological induced inflammation[43]. Furthermore, flavonoids seem to be safe[71] and while flavonoids have low bioavailability, already several studies have investigated different delivery systems to solve this problem (reviewed here). However, more research needs to be conducted, especially into food allergy and into the effects of flavonoids in humans.

References

1. Sicherer, S. H. & Sampson, H. a. Food allergy. *J. Allergy Clin. Immunol.* **125,** S116–25 (2010).
2. Ruiter, B. & Shreffler, W. G. The role of dendritic cells in food allergy. *J. Allergy Clin. Immunol.* **129,** 921–8 (2012).
3. Holgate, S. T. The epidemic of allergy and asthma. *Nature* **402,** B2–4 (1999).
4. Kindt T. J., Osborne B. A., G. R. A. *Kuby Immunology*. 546 (W. H. Freeman & Company, 2006).
5. Barnes, P. J. Pathophysiology of allergic inflammation. *Immunol. Rev.* **242,** 31–50 (2011).
6. Islam, S. a & Luster, A. D. T cell homing to epithelial barriers in allergic disease. *Nat. Med.* **18,** 705–15 (2012).
7. Kaplan, a P. Chemokines, chemokine receptors and allergy. *Int. Arch. Allergy Immunol.* **124,** 423–31 (2001).
8. Velazquez, J. R. & Teran, L. M. Chemokines and their receptors in the allergic airway inflammatory process. *Clin. Rev. Allergy Immunol.* **41,** 76–88 (2011).
9. Pease, J. E. Targeting chemokine receptors in allergic disease. *Biochem. J.* **434,** 11–24 (2011).
10. Tanaka, T. & Takahashi, R. Flavonoids and asthma. *Nutrients* **5,** 2128–43 (2013).
11. Hämäläinen, M., Nieminen, R., Vuorela, P., Heinonen, M. & Moilanen, E. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory eff. *Mediators Inflamm.* **2007,** 45673 (2007).
12. Lambrecht, B. N. & Hammad, H. The airway epithelium in asthma. *Nat. Med.* **18,** 684–92 (2012).
13. Dupont, C. Food allergy: recent advances in pathophysiology and diagnosis. *Ann. Nutr. Metab.* **59 Suppl 1,** 8–18 (2011).
14. Lambrecht, B. N. & Hammad, H. Asthma: the importance of dysregulated barrier immunity. *Eur. J. Immunol.* (2013). doi:10.1002/eji.201343730

15. Xiao, C. *et al.* Defective epithelial barrier function in asthma. *J. Allergy Clin. Immunol.* **128,** 549–56.e1–12 (2011).

16. Ohshima, Y. Mucosal immunity and the onset of allergic disease. *Allergol. Int.* **62,** 279–89 (2013).

17. Gonzalo, J. *et al.* Coordinated role of mast cells and T cells in Allergic Mucosal Inflammation: Critical Role of the CC Chemokine Ligand 1:CCR8 Axis. *J. Immunol.* **179,** 1740–1750 (2007).

18. Ito, T., Carson, W. F., Cavassani, K. a, Connett, J. M. & Kunkel, S. L. CCR6 as a mediator of immunity in the lung and gut. *Exp. Cell Res.* **317,** 613–9 (2011).

19. Lee, E.-J., Ji, G.-E. & Sung, M.-K. Quercetin and kaempferol suppress immunoglobulin E-mediated allergic inflammation in RBL-2H3 and Caco-2 cells. *Inflamm. Res.* **59,** 847–54 (2010).

20. Gupta, S. *et al.* Intravascular inactivation of CCR5 by n-Nonanoyl-CC chemokine ligand 14 and inhibition of allergic airway inflammation. *J. Leukoc. Biol.* **83,** 765–73 (2008).

21. Mikhak, Z. *et al.* Contribution of CCR4 and CCR8 to antigen-specific T(H)2 cell trafficking in allergic pulmonary inflammation. *J. Allergy Clin. Immunol.* **123,** 67–73.e3 (2009).

22. Afshar, R. *et al.* Compartmentalized chemokine-dependent regulatory T-cell inhibition of allergic pulmonary inflammation. *J. Allergy Clin. Immunol.* **131,** 1644–52 (2013).

23. Heiman, A. S., Abonyo, B. O., Darling-Reed, S. F. & Alexander, M. S. Cytokine-stimulated human lung alveolar epithelial cells release eotaxin-2 (CCL24) and eotaxin-3 (CCL26). *J. Interferon Cytokine Res.* **25,** 82–91 (2005).

24. Fulkerson, P. C. *et al.* A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 16418–23 (2006).

25. Costa, M. F. S. *et al.* CCL25 induces $\alpha_4\beta_7$ integrin-dependent migration of IL-17$^+$ γδ T lymphocytes during an allergic reaction. *Eur. J. Immunol.* **42,** 1250–60 (2012).

26. Tubo, N. J. *et al.* A systemically-administered small molecule antagonist of CCR9 acts as a tissue-selective inhibitor of lymphocyte trafficking. *PLoS One* **7,** e50498 (2012).

27. Mellado, M. *et al.* Chemokine Receptor 2 Blockade Prevents Asthma in a Cynomolgus Monkey Model. **324,** 769–775 (2008).

28. Bellinghausen, I. *et al.* Enhanced production of CCL18 by tolerogenic dendritic cells is associated with inhibition of allergic airway reactivity. *J. Allergy Clin. Immunol.* **130,** 1384–93 (2012).

29. Tian, L. *et al.* The CKLF1-C19 peptide attenuates allergic lung inflammation by inhibiting CCR3- and CCR4-mediated chemotaxis in a mouse model of asthma. *Allergy* **66,** 287–97 (2011).

30. Komai, M. *et al.* A Novel CC-Chemokine Receptor 3 Antagonist, Ki19003, Inhibits Airway Eosinophilia and Subepithelial/Peribronchial Fibrosis Induced by Repeated Antigen Challenge in Mice. *J. Pharmacol. Sci.* **112,** 203–213 (2010).

31. Sato, T. *et al.* Inhibitory effect of the new orally active CCR4 antagonist K327 on CCR4+CD4+ T cell migration into the lung of mice with ovalbumin-induced lung allergic inflammation. *Pharmacology* **84,** 171–82 (2009).

32. Wang, L. *et al.* Antagonism of chemokine receptor CCR8 is ineffective in a primate model of asthma. *Thorax* **68,** 506–12 (2013).

33. Suzaki, Y. *et al.* A small-molecule compound targeting CCR5 and CXCR3 prevents airway hyperresponsiveness and inflammation. *Eur. Respir. J.* **31,** 783–9 (2008).

34. Daubeuf, F. *et al.* An antedrug of the CXCL12 neutraligand blocks experimental allergic asthma without systemic effect in mice. *J. Biol. Chem.* **288,** 11865–76 (2013).

35. Elsner, J., Escher, S. E. & Forssmann, U. Chemokine receptor antagonists: a novel therapeutic approach in allergic diseases. *Allergy* **59,** 1243–58 (2004).

36. Huang, C.-H. *et al.* The natural flavonoid apigenin suppresses Th1- and Th2-related chemokine production by human monocyte THP-1 cells through mitogen-activated protein kinase pathways. *J. Med. Food* **13,** 391–8 (2010).

37. Huang, C.-H. *et al.* Natural flavone kaempferol suppresses chemokines expression in human monocyte THP-1 cells through MAPK pathways. *J. Food Sci.* **75,** H254–9 (2010).

38. Kim, H.-H., Bae, Y. & Kim, S.-H. Galangin attenuates mast cell-mediated allergic inflammation. *Food Chem. Toxicol.* **57,** 209–16 (2013).

39. Zuercher, a W., Holvoet, S., Weiss, M. & Mercenier, a. Polyphenol-enriched apple extract attenuates food allergy in mice. *Clin. Exp. Allergy* **40,** 942–50 (2010).

40. Huang, W.-C. & Liou, C.-J. Dietary acacetin reduces airway hyperresponsiveness and eosinophil infiltration by modulating eotaxin-1 and th2 cytokines in a mouse model of asthma. *Evid. Based. Complement. Alternat. Med.* **2012,** 910520 (2012).

41. Nanua, S. *et al.* Quercetin blocks airway epithelial cell chemokine expression. *Am. J. Respir. Cell Mol. Biol.* **35,** 602–10 (2006).

42. Kempuraj, D. *et al.* Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. *Br. J. Pharmacol.* **145,** 934–44 (2005).

43. Castellani, M. L. *et al.* Inhibitory effect of quercetin on tryptase and MCP-1 chemokine release, and histidine decarboxylase mRNA transcription by human mast cell-1 cell line. *Neuroimmunomodulation* **13,** 179–86 (2006).

44. Gong, J., Shin, D., Han, S., Kim, J. & Kang, Y. Kaempferol Suppresses Eosionphil Infiltration and Airway Inflammation in Airway Epithelial Cells and in Mice with Allergic Asthma 1 , 2. (2012). doi:10.3945/jn.111.150748.shown

45. Wu, M.-Y., Hung, S.-K. & Fu, S.-L. Immunosuppressive effects of fisetin in ovalbumin-induced asthma through inhibition of NF-κB activity. *J. Agric. Food Chem.* **59,** 10496–504 (2011).

46. Goh, F. Y. *et al.* Fisetin, a bioactive flavonol, attenuates allergic airway inflammation through negative regulation of NF-κB. *Eur. J. Pharmacol.* **679,** 109–16 (2012).

47. Bao, Z. *et al.* Inhibition of airway inflammation, hyperresponsiveness and remodeling by soy isoflavone in a murine model of allergic asthma. *Int. Immunopharmacol.* **11,** 899–906 (2011).

48. Toledo, a C. *et al.* Flavonone treatment reverses airway inflammation and remodelling in an asthma murine model. *Br. J. Pharmacol.* **168,** 1736–49 (2013).

49. Choi, Y. H. *et al.* Silibinin attenuates allergic airway inflammation in mice. *Biochem. Biophys. Res. Commun.* **427,** 450–5 (2012).

50. Ziegler, S. F. Thymic stromal lymphopoietin and allergic disease. *J. Allergy Clin. Immunol.* **130,** 845–52 (2012).

51. Masilamani, M. *et al.* Soybean isoflavones regulate dendritic cell function and suppress allergic sensitization to peanut. *J. Allergy Clin. Immunol.* **128,** 1242–1250.e1 (2011).

52. Yum, M. K., Jung, M. Y., Cho, D. & Kim, T. S. Suppression of dendritic cells' maturation and functions by daidzein, a phytoestrogen. *Toxicol. Appl. Pharmacol.* **257,** 174–81 (2011).

53. Wei, J., Bhatt, S., Chang, L. M., Sampson, H. a & Masilamani, M. Isoflavones, genistein and daidzein, regulate mucosal immune response by suppressing dendritic cell function. *PLoS One* **7,** e47979 (2012).

54. Lin, M.-K. *et al.* Kaempferol from Semen cuscutae attenuates the immune function of dendritic cells. *Immunobiology* **216,** 1103–9 (2011).

55. Huang, R.-Y. *et al.* Immunosuppressive effect of quercetin on dendritic cell activation and function. *J. Immunol.* **184,** 6815–21 (2010).

56. Miyake, Y. *et al.* Soy, isoflavones, and prevalence of allergic rhinitis in Japanese women: the Osaka Maternal and Child Health Study. *J. Allergy Clin. Immunol.* **115,** 1176–83 (2005).

57. Kawai, M. *et al.* Effect of enzymatically modified isoquercitrin, a flavonoid, on symptoms of Japanese cedar pollinosis: a randomized double-blind placebo-controlled trial. *Int. Arch. Allergy Immunol.* **149,** 359–68 (2009).

58. Kalhan, R. *et al.* A mechanism of benefit of soy genistein in asthma: inhibition of eosinophil p38-dependent leukotriene synthesis. *Clin. Exp. Allergy* **38,** 103–12 (2008).

59. Bime, C., Wei, C. Y., Holbrook, J., Smith, L. J. & Wise, R. A. Association of dietary soy genistein intake with lung function and asthma control: a post-hoc analysis of patients enrolled in a prospective multicentre clinical trial. *Prim. Care Respir. J.* **21,** 398–404 (2012).

60. Watson, R. R. *et al.* Oral administration of the purple passion fruit peel extract reduces wheeze and cough and improves shortness of breath in adults with asthma. *Nutr. Res.* **28,** 166–71 (2008).

61. Mignet, N., Seguin, J. & Chabot, G. G. Bioavailability of polyphenol liposomes: a challenge ahead. *Pharmaceutics* **5,** 457–71 (2013).

62. Chen, J. U. N., Lin, H. & Hu, M. Metabolism of Flavonoids via Enteric Recycling : Role of Intestinal Disposition. **304,** 1228–1235 (2003).

63. Yuan, Z. *et al.* Liposomal quercetin efficiently suppresses growth of solid tumors in murine models. *Clin. Cancer Res.* **12,** 3193–9 (2006).

64. Seguin, J. *et al.* Liposomal encapsulation of the natural flavonoid fisetin improves bioavailability and antitumor efficacy. *Int. J. Pharm.* **444,** 146–54 (2013).

65. Kang, M. J. *et al.* Pep-1 peptide-conjugated elastic liposomal formulation of taxifolin glycoside for the treatment of atopic dermatitis in NC/Nga mice. *Int. J. Pharm.* **402,** 198–204 (2010).

66. Barras, a *et al.* Formulation and characterization of polyphenol-loaded lipid nanocapsules. *Int. J. Pharm.* **379,** 270–7 (2009).

67. Rogerio, A. P. *et al.* Anti-inflammatory effect of quercetin-loaded microemulsion in the airways allergic inflammatory model in mice. *Pharmacol. Res.* **61,** 288–97 (2010).

68. Isoda, H. *et al.* Antiallergic effect of Picholine olive oil-in-water emulsions through β-hexosaminidase release inhibition and characterization of their physicochemical properties. *J. Agric. Food Chem.* **60,** 7851–8 (2012).

69. Ragelle, H. *et al.* Nanoemulsion formulation of fisetin improves bioavailability and antitumour activity in mice. *Int. J. Pharm.* **427,** 452–9 (2012).

70. Pralhad, T. & Rajendrakumar, K. Study of freeze-dried quercetin-cyclodextrin binary systems by DSC, FT-IR, X-ray diffraction and SEM analysis. *J. Pharm. Biomed. Anal.* **34,** 333–9 (2004).

71. Weng, Z. *et al.* Quercetin is more effective than cromolyn in blocking human mast cell cytokine release and inhibits contact dermatitis and photosensitivity in humans. *PLoS One* **7,** e33805 (2012).